REVUE SUISSE DE ZOOLOGIE
Tome 59, nº 22. — Septembre 1952.

AUS DER ZOOPHYSIOLOGISCHEN ABTEILUNG DES ZOOLOGISCHEN
INSTITUTS DER UNIVERSITÄT BERN.

Histostatic and cytostatic effects of some Amino Ketones upon Tail Regeneration in *Xenopus* Larvae

by

H. R. DETTELBACH

with 19 text-figures.

TABLE OF CONTENTS

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

II. MATERIALS AND METHODS.

- A. *Materials.*
- B. *Amputation and Chemical Treatment of the Larvae.*
- C. *Measurements.*
- D. *Fixing and Staining.*

III. EXPERIMENTAL RESULTS.

- A. *Phases of Normal Regeneration.*
- B. *Morphological Types of Reaction to Treatment with Amino Ketones.*
  - 1. General Effects.
  - 2. The Types of Response to Treatment.
  - 3. Suppression of Notochord and Spinal Cord.
  - 4. Persistance of Effects.
- C. *Measureable Effects Upon Growth and Regeneration.*
  - 1. The degree of Inhibition.

2. Susceptibility and Threshold.
3. Sensitivity as a Function of the Time of Treatment.
4. Pre-treatment.
5. Effect of Temperature Variation.
6. Toxicity and Effective Range of Concentrations.

D. *Histostatic and Cytostatic Effects Upon Regenerating Tissues.*
1. Epidermis.
2. Basal Membrane.
3. Mesenchyme Area.
4. Notochord and Sheath.
5. Spinal Cord.
6. Mitoses.
7. Nuclear Size Variations.
8. Nucleolar Size Variations.

IV. THE EFFECTS OF AMINO KETONES UPON EMBRYONIC CHICK FIBROBLASTS IN VITRO.

A. *Methods and Materials.*

B. *Effects upon Cell Division.*

C. *Effects upon Cell Migration.*

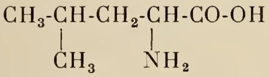
V. SUMMARY.

VI. LITERATURE.

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

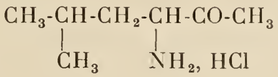
Tail regeneration in anuran larvae may be inhibited through the use of chemical agents. LEHMANN, BERNHARD, HADORN and LÜSCHER (1945) were the first to achieve a total suppression of tail regeneration through the application of colchicine, and LÜSCHER (1946*a*) and BERNHARD (1947) have made use of this fact to study the biological action of colchicine in detail. LÜSCHER found that this substance produces its main effects upon regeneration by inducing large numbers to cells to enter into mitosis prematurely, whereupon they are blocked in the metaphase and consequently degenerate. Because of this action colchicine may be classified as an antimitotic with a pronounced nuclear destructive action upon cells.

Recently Erlenmeyer and Lehmann (1949), Erlenmeyer and Kühne (1949), and Lehmann, Bretscher, Kühne, Sorkin, Erne and Erlenmeyer (1950) reported that some of the newly synthesized α-amino ketones are biologically active and capable of significantly inhibiting regeneration of the *Xenopus* larva tail. Of the several amino ketones, those which are structurally similar to α-leucine (Erlenmeyer, 1948) with either a methyl or ethyl group substituted for the end OH group proved most active. (Lehmann et. al., *op. cit.*, p. 1219.)



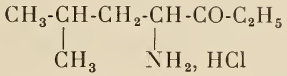
$CH_3\text{-}\underset{\displaystyle CH_3}{CH}\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{CH}\text{-}CO\text{-}OH$

α-leucine



$CH_3\text{-}\underset{\displaystyle CH_3}{CH}\text{-}CH_2\text{-}\underset{\displaystyle NH_2,\ HCl}{CH}\text{-}CO\text{-}CH_3$

amino ketone 5



$CH_3\text{-}\underset{\displaystyle CH_3}{CH}\text{-}CH_2\text{-}\underset{\displaystyle NH_2,\ HCl}{CH}\text{-}CO\text{-}C_2H_5$

amino ketone 9

Details of the method of synthesis and physical, and some of the chemical properties of these substances are given in the above mentioned papers and also in Kühne (1950) (see also v. Euler, 1950).

As Erlenmeyer and Lehmann pointed out *(op. cit.)*, the amino ketones differ sharply in their action upon regenerating tissues from substances such as colchicine. They reported few but apparently normal mitoses in the regenerating tissues which led to the suggestion that these substances had a more histostatic action than an antimitotic one. Further, the effective range of concentrations was found to be quite wide in comparison to the known antimitotics. Addition of α-leucine to the solutions had no effect upon the action of the substances. As mentioned in the papers of Erlenmeyer and Lehmann *(op. cit.)* and Lehmann et. al. (*op. cit.*, p. 1221), the amino ketones are strong reducing agents, relatively stable in acid solutions, but oxidized in alkaline solutions by the oxygen of the air. Lehmann and Weber (unpublished, communicated with permission of the authors) have since determined that the amino ketones are more effective if used in a buffered solution of pH 7.2 and considerably less effective in a buffered solution at a pH of 5.5. Aebi (unpublished) has tested the amino ketones in the Warburg apparatus and found that they both take up oxygen at a rate which increases as the pH of the medium is increased. He also found that amino ketone 5 takes up oxygen 3 to 4 times as fast as does ketone 9 and that both take up 2 atoms of oxygen per molecule. In view of these facts it seems evident that the amino ketones themselves are *not* responsible for the inhibition of regeneration but that some oxidation product is the active agent. The different rates of oxygen uptake may also be considered the basis for the differences in effects caused by the two amino ketones (e.g. nuclear size variations and duration of effects). In spite of the above, inhibition of regeneration by our method of amino ketone treatment is consistent and the results reproduceable.

These findings indicate that the main effect of the amino ketones or their oxidation products upon the regenerating tissues is a histostatic one, and relatively few signs of severe cellular damage are to be found. (See also Lehmann and Dettelbach, 1952.)

The aim of the present paper is to extend the above mentioned results and to examine in detail the mode of action of the amino ketones upon regeneration and growth of *Xenopus* larvae. Measurement of the regenerating larval tail under different conditions of temperature, time of treatment, pre-treatment, etc., were combined with a histological and cytological examination of fixed tissues. By these methods and by testing the effects of the ketones upon embryonic chick tissues *in vitro*, results were obtained concerning the mode of action of these substances.

I would like to take this opportunity to express my thanks and appreciation to Professor F. E. Lehmann for suggesting this problem to me and for his continual interest and many suggestions during the course of the work.

I am also indebted to Dr. A. Bretscher for his help with the statistical analysis. I also had the opportunity to read the manuscript by F. E. Lehmann and A. Bretscher "Wirkungsanalyse regenerationshemmender Stoffe mit Hilfe statistischer Methoden." (1952).

II. MATERIALS AND METHODS

A. Materials.

The larvae of *Xenopus laevis* are readily obtainable during the whole year in practically unlimited numbers. In principle the method of

Ochsé (1948) is followed with some modifications (Andres, Bretscher, Lehmann, Roth 1949). Copulation and egg laying may be brought about relatively easily by the injection of gonadotropic hormones (Ochsé, *op. cit.*, p. 63). As the larvae are only very slightly pigmented, observation of the transparent organs and tissues, and especially the tail, is simple when the animals are narcotized with MS 222 (Sandoz, Basel)[1]. The keeping of *Xenopus* and the raising of larvae has been carried on successfully in this institution for a number of years. The larvae after hatching are fed exclusively with finely ground nettle powder *(Urtica)* upon which they thrive and grow rapidly. They are kept in round enameled pans at a constant temperature of 20° to 24° C in a thermostat.

B. Amputation and Chemical Treatment of the Larvae.

For each experimental series tadpoles from the same egg batch were chosen which varied in size by not more than 4 mm and 5 animals were used per container at the various concentrations. Tadpoles to be treated were first narcotized in MS 222 (Sandoz) 1: 7000, at a temperature of about 20° C for a period between 5 and 10 minutes. The narcotized animals were then selected on paraffined millimeter paper according to size. On animals found to be of a size within the limits set for the experiment, 4 mm were amputated from the tip of the tail with a fine scalpel. A period of 5 to 10 minutes was then allowed for the animals to recover in distilled water. After that they were transferred to the already prepared test solutions. Five animals were placed in each glass containing 150 cc of solution. All selection and division of the animals was done completely at *random*. The containers with the tadpoles were placed in a large thermostatically controlled cabinet where temperature was maintained at 18° ± 1° C. In order to be sure that enough substance was present in the solutions to affect all five of the tadpoles and to affect them to the maximum, parallel experiments were conducted in which one series of ketone solutions contained five animals per 150 cc container and one series contained one animal per 150 cc container. The results of these experiments show that there is no significant difference in the amount of inhibition when only one animal per 150 cc is used instead of five. All subsequent experiments were therefore conducted with five animals per container.

It was desired to obtain a quantitative expression of the influence of the amino ketones upon regeneration. All of the experiments were therefore performed with a graded series of amino ketone solutions of geometrically decreasing concentration in distilled water (e.g. 1: 1000, 1: 2000, 1: 4000, 1: 8000, 1: 16000, 1: 32000, 1: 64000, 1: 125000, 1: 250000, 1: 500000, 1: 1000000, and distilled water).

---

[1] A detailed study of the development and morphology of the *Xenopus* larva has recently been made by Weisz (1945).

All water used in these experiments was glass distilled. Ordinary tap water in Berne is toxic because of its chlorine content. Tadpoles of *Xenopus* are able to live in the distilled water very well for periods much longer than 10 days. The pre-experimental conditions of feeding, temperature, etc. may determine to a large degree the regenerative capacity of a given group of larvae (Zeleny, 1917; Lüscher, 1946 *a*). Even when conditions have been optimal and the same for all larvae, different batches still show variations in their capacity for regeneration. Temperatures of less than 10° C produce a large and permanent reduction of the regenerative capacity. Temperatures of 25° to 28° C cause an acceleration of regeneration to about double that at 18° C. Insufficient feeding prior to amputation also leads to a reduction or failure of regeneration. Greater amounts of tissue material amputated cause an increase in regeneration rate, and re-amputation also causes an acceleration. (Lüscher, *loc. cit.*, p. 472; Zeleny, *op. cit.*) The size of the animals has only a small influence upon their regeneration provided the larvae are between 15 and 40 mm in length. It was attempted to eliminate or control as many of these extrinsic factors as possible by treating all larvae in exactly the same manner and by standardizing the experimental method.

C. Measurement of Regenerating Tails.

Rötheli (1947, unpublished) has shown that the increase of length of the regenerating *Xenopus* tail is directly proportional to the increase in surface area. Thus measurement of length alone is an expression of the area regenerated. For this reason only the length of the regenerating tail was measured. Daily measurement of the regenerating tail had considerable deleterious effects and raised the incidence of mortality to an extent that invalidated some of the experiments. Limiting the number of measurements to the 5th and 10th days eliminated this. It is also possible to measure on the 5th and 10th days tails which have been reduced by the chemical treatment. Measurement was done directly with an ocular micrometer in tenths of millimeters by adjustment of the extensible microscope barrel (Lüscher 1946 *a*). Illumination was through a heat absorbent filter. The regenerating tail was measured from the point of amputation which is marked by an indentation in the notochord and by the different appearance of the regenerated epidermis. No narcosis was used. Slight variations made in the procedure at times are noted in the text.

D. Fixing and staining.

(See Lüscher, 1946 *a.*)

III. EXPERIMENTAL RESULTS

A. Phases of Normal Regeneration.

The chemically caused changes in the regeneration process only have meaning when they are compared with the normal regeneration. Lüscher (1946 *a*) has shown that such changes of the tail length are measureable and may be compared with untreated animals. Noticeable morphological changes may be classified under a few types as Lüscher has done (p. 477). The various reaction types are described below followed by a survey of the quantitative inhibition of tail regeneration.

Normal regeneration of the Amphibian tail in general (Abeloos, 1932; Godlewski, 1928; Harrison, 1898; et. al.) and that of *Xenopus laevis* in particular (Lüscher, 1946 *a*, 1946 *b*) has already been given. The phases of normal regeneration (photographed by Lüscher 1946 *a*) may be divided into three phases.

Phase 1. *Wound healing and formation of small tissue specific regeneration blastemas by cell migration*[1]. During *this phase*, which *lasts about three days*, *autolysis and activation of the tissues takes place.*

Phase 2. *Growth of the regeneration blastemas without differentiations. Cell division in the stump begins* during this phase which starts on about the third day and lasts till about the sixth day.

Phase 3. *Growth and differentiation of the regenerating tissues*, beginning on about the fourth day and partly concurrent with the preceeding phase. *Active cell division and increase in the length of the regenerating tail during this phase.* (See also Rosin, 1946, p. 184.)

As will be shown, the amino ketones act strongly upon phase 1, while phase 2 and 3 are less affected.

---

[1] Godlewski (1928), Arey (1932), Zeleny (1916), Butler (1933), Waechter (1949), and others, have all reported that the first step in wound healing in amphibians is a migration of epidermal cells to the wound surface, and that cell division first begins usually on about the 4th day. Wigglesworth (1937, p. 368) found a similar condition during wound healing in insects. This sequence of events probably holds true for all wound healing.

B. MORPHOLOGICAL TYPES OF REACTION TO TREATMENT WITH AMINO KETONES.

1. *General Effects.*

The effects of treatment with stronger solutions of the amino ketones are not limited to the inhibition of regeneration. The tadpoles in the stronger solutions (1: 16000 and stronger) show a distinct contraction of the pigment cells which makes the tadpoles pale in contrast to the controls. SCHEREMETIEVA and BRUNST (1933, p. 780) also found contraction of the pigment cells in larvae of *Pelobates fusca* after X-raying. LÜSCHER (1946 *b*, p. 693) found that colchicine treatment causes an expansion of the pigment cells of the skin which may last as long as one month after treatment. Amino ketone treated animals are considerably more sluggish in their movements and often refrain from taking their typical 45° angle of inclination. The usually constant tail movement is also often lost.

The amino ketones cause, like colchicine (LÜSCHER, 1946 *a*, p. 480), a *statistically significant reduction of the total length* over a period of 10 days at a concentration of 1: 32000. In one experiment control tadpoles were 23.4 mm on the 5th day and 22.3 mm on the 10th day, while amino ketone treated tadpoles of the same initial length were 21.8 on the 5th day and 20.6 mm on the 10th day. This reduction usually amounts to 8 to 10% of the control length and is quite evident without amputation which demonstrates that the substances are taken up other than through the wound surface only. Treatment at a concentration of middle strength however limits the effects practically wholly to the regenerating tail extremity. Solutions of 1: 64000, for example, cause consistent and definite inhibition of regeneration while any general effects are at a minimum and recovery is usually complete within a short time.

2. *The Types of Response to Treatment* (see figure 1).

The response to amino ketone treatment after amputation may be classified into three main types. These reaction types correspond to those found by LÜSCHER (1946 *a*) after treatment with colchicine and his numbering is maintained here.

Type I (highest realization grade) is the weakest reaction to treatment and composed of two sub-types. Type I *a* is found with the greatest frequency and may be interpreted as simply *delayed normal regeneration.* Axial organs show good growth on the 10 the day and growth seems to be progressing well at this point. The length of the regenerate on the 10th day of type I *a* extends over the whole range from the maximal of type II up to control lengths. The extent of inhibition depends upon the concentration at which treated and the regenerative capacity of the particular group of animals as shown by the controls. The difference between type I *a* and most controls is mainly a matter of degree.

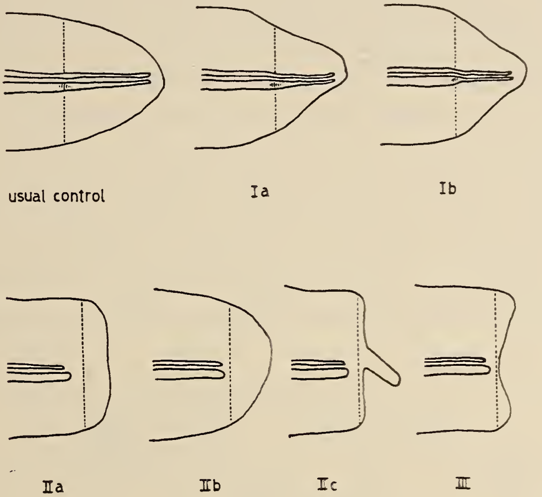


Fig. 1.

Above are illustrated the principal types of tail regeneration of *Xenopus* larvae. Note that types II and III show suppression of axial organ regeneration. The broken lines indicate the place of amputation.

Type I *b* response shows *the weakest regeneration in which the notochord and spinal cord take part.* The notochord is formed as a thin extension from the place of amputation and shows a development that is poor when compared with the controls. The point of amputation is clearly marked by a sharp difference in the diameter of the notochord. Regenerated tails of this type are usually cone shaped and are limited to a length of 1.1 to 1.3 mm.

Type II (second realization grade) is a *limited regeneration* of the tail tip *with suppression of the notochord and spinal cord.* Included under type II are three sub-types representing varying degrees of regeneration without axial organs. Regeneration of this type is principally epidermal with some mesenchyme taking part. The epidermal cells in such regenerates are normal in appearance but are not as well organized as in control epidermis.

Type II *a* is normal wound healing plus the formation of a small mound of tissue composed of muscle and connective tissue around and over the notochord cap. At the end of ten days of treatment this type of response may reach a length of 0.3 to 0.5 mm. Type II *b* represents a continuation of type II *a* and leads to the formation of a rounded fin-like structure without axial organs. It is usually 0.5 to 0.6 mm in length. Type II *c* is the most abberrant of group II and is composed of thin growths that are often bent and irregular in shape and lack axial organs. Regeneration of type II *c* is quite variable in length and in single cases may attain 1.0 mm or more at the end of ten days (figure 2).

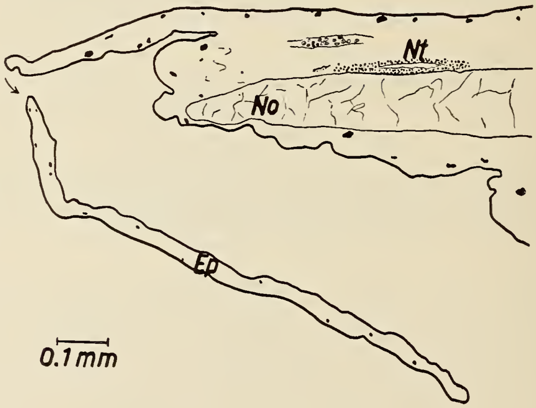


Fig. 2.

Case of extreme epidermal regeneration with suppression of axial organs (Type II *c*) after treatment with ketone 5, 1: 8000 for 10 days. Length after fixing 3.3 mm. Sagittal section. 80 X. No = Notochord, Nt = Neural tube, Ep = Epidermal Regenerate.

Type III (first realization grade and strongest reaction) is a *complete inhibition of regeneration* with only wound healing. Animals showing this type of response show no progress during the whole experiment and the condition on the 10th day is essentially the same as that seen shortly after amputation. The covering over the stump is limited to 0.2 mm in thickness and is simply an epidermal layer. This degree of inhibition is seldom in appearance and is seen only at concentrations of 1: 8000 and stronger. The frequency of appearance of this type increases with increasing concentration.

Type I is found in most animals after 10 days treatment at concentrations of 1: 8000 and more dilute. The frequency of appearance of type II and III increases in most groups at 1: 8000 as the treatment becomes stronger but there is a very large variation from group to group. In some batches type II and III appear at a concentration of 1: 16000, while in others they are only found at concentrations of 1: 2000 or more. In a series of 9 experiments in which concentrations of 1: 16000—1: 2000 were included, types II and III were found 46 times out of the total 225 animals treated. It was found impossible to establish any difference in the frequency of types II and III after ketone 5 and after ketone 9 treatment. Type II is generally from three to five times more frequent than type III. Animals showing type II or III reaction are unable to recover from the effects of treatment even under optimal conditions after the 10th day while type I continues to grow and eventually overtakes the controls after some twenty days or more (see figure 3). It appears that the suppression of the axial organs exerts a limiting effect upon the amount of tail regeneration possible. In figure 1 are shown diagrammatically the various regeneration types.

It seems probable that the three types described are examples of distinct " realization grades " [1]. As the intensity of treatment is increased, tail regeneration becomes progressively less until a certain minimal size is reached. When the concentration is increased beyond this point a degradation stage is reached in which the notochord and spinal cord fail to regenerate and only a fin-like

---

[1] For the general treatment of the concept of " realization grades " see F. E. LEHMANN, 1948, Archiv JULIUS KLAUS-Stiftung. 23: 568-573.

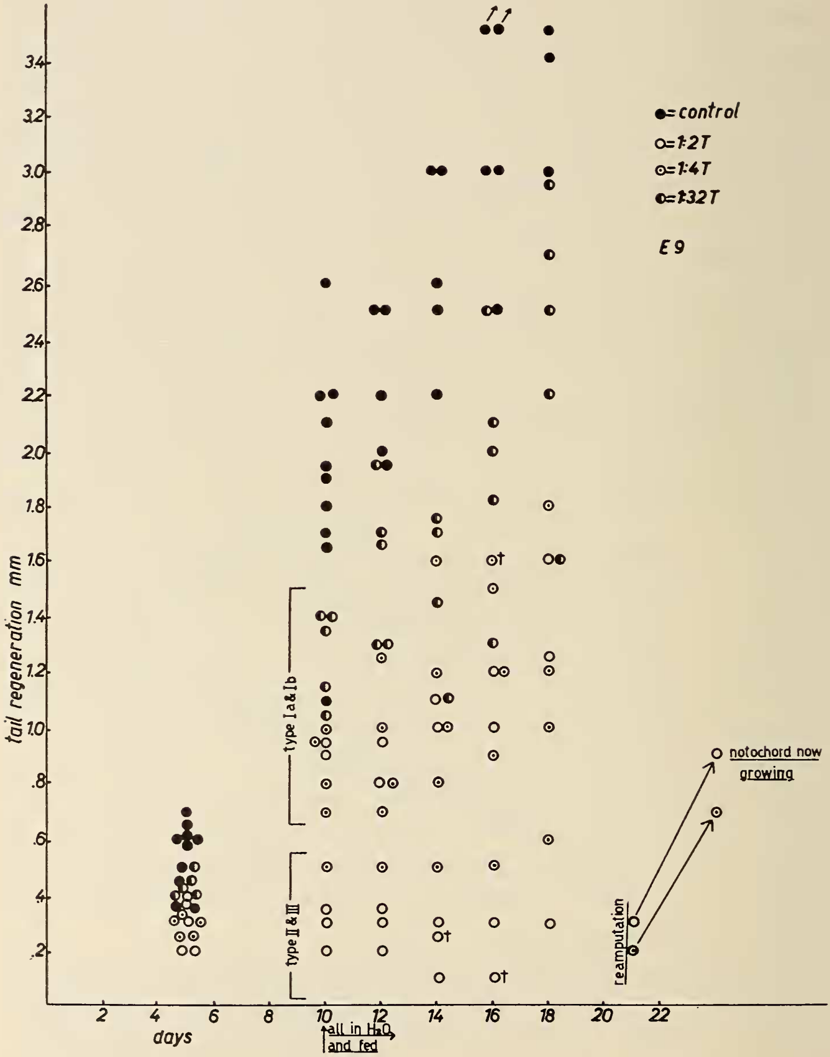


Fig. 3.

Graph of part of one regeneration experiment of ketone 9 treated larvae. Measurements were continued over 20 days to show the course of regeneration. Note that the larvae in which regeneration has been blocked for 20 days begin at once to regenerate when the tail tip is reamputated.

tail tip is formed (see Dalcq, 1947, pp. 114-115. Principle of Minimal Area of a Blastema). This is a "realization grade" below the normal. By again increasing the concentration of the amino ketones, the third realization grade is reached which is characterized by the lack of even a fin regenerate.

The production of realization grades has been found in other forms and by other means as well: e.g. Puckett (1936), reduction of the number of digits and limb parts in Amblystoma after X-raying; Bretscher, reduction of the number of toes in *Xenopus* (1947) and suppression of polydactyly in the chick (1950) both after colchicine treatment; Bretscher and Tschumi (1951), reduction of the number of digits and parts of the leg of *Xenopus* after colchicine and mustard gas treatment; Andres (1948) for the development of the amphibian labyrinth; Dalcq *(op. cit.)* for brain and notochord of amphibians, etc.). Common to all appears to be the *reduction in the number of parts or organs* with increasing degradation and *not a harmonious reduction of the size of the whole anlage* (Lehmann, 1948, p. 569).

3. *The role of the Notochord Blastema in the Formation of Realization Grades.*

As a result of strong treatment the axial organs of the tail may be completely prevented from regenerating (see foregoing section). (Type I and II, figure 1.) In this case wound healing takes place with a limited amount of epidermal regeneration to form a fin-like structure devoid of support. Such suppressed regeneration is found after treatment at concentrations of 1: 8000 and stronger but not in all animals which have been so treated. At concentrations stronger than 1: 2000 almost 100% of the surviving tadpoles show this type of response.

The different realization grades give an indication of the degree of sensitivity of the various components of the regenerating tail. Within a large concentration range the regeneration of all organ blastemas taking part is first slowed without the appearance of qualitative differences. After stronger reaction, the volume of the regenerating neural tube, and especially *the notochord*, are clearly too small (I *b*). When the regeneration of the notochord and neural tube are suppressed only a relatively small epidermal tail fin is formed. (II) At still stronger reaction the regeneration of the epidermis is also suppressed. (III) These realization grades appear after colchicine treatment (Lüscher, 1946 *a*) in a similar way as after amino ketone treatment. The findings indicate that the graduated sensitivity of the different regeneration blastemas is responsible for the realization grades.

Scheremetieva and Brunst (1933, 1936) have found that regenerative capacity in the X-rayed tail of *Pelobates* was lost in the following order: *notochord*, neural tube and musculature, gelatinous connective tissue, blood vessels, epidermis.

This order seems to agree essentially with that found after treatment with the amino ketones. All the results in the literature (see also Avel, 1932; Okada, 1936; and Helff, 1937) and the results of colchicine and amino ketone treatment of *Xenopus laevis* point to *the notochord blastema as the chief factor* in tail regeneration. Because the notochord blastema is also the most sensitive of those taking part, the first realization grades (I *b* and II) depend upon the partial or total suppression of this blastema. In the colchicine experiments the suppression of the notochord blastema is caused by the loss of pre-mitotic cells. The action of the amino ketones on the notochord blastema is made clear by histological examination (Section D).

It is improbable that all the cells of the notochord blastema are suppressed by the chemical treatment. The question may therefore be asked if there is not a certain minimal size necessary for a blastema which must be attained before regeneration is possible. If the number of cells taking part in the regeneration of the notochord or epidermis is insufficient, a certain, as yet unknown, physiological threshold (see also Dalcq, *loc. cit.*) is not reached and the regeneration of the blastema in question fails completely.

4. *Persistance of Effects.*

Larvae in which notochord and spinal cord have been prevented from regenerating show no subsequent recovery at least for periods as long as two months after treatment (longest period examined). Upon re-amputation of only a very small amount of tail tissue (with the cut passing through all of the tissues of the tail). Regeneration begins and leads to formation of a well developed tail tip (see figure 3).

It appears that if the capacity for regeneration has not been permanently suppressed by chemical treatment, an additional regeneration stimulus is necessary. Once regeneration has begun, it continues normally and the larvae eventually pass through a normal metamorphosis. Figure 3 shows the course of growth in an experiment in which some animals were first completely inhibited. Note that upon re-amputation regeneration begins and at the same rate as that of the controls.

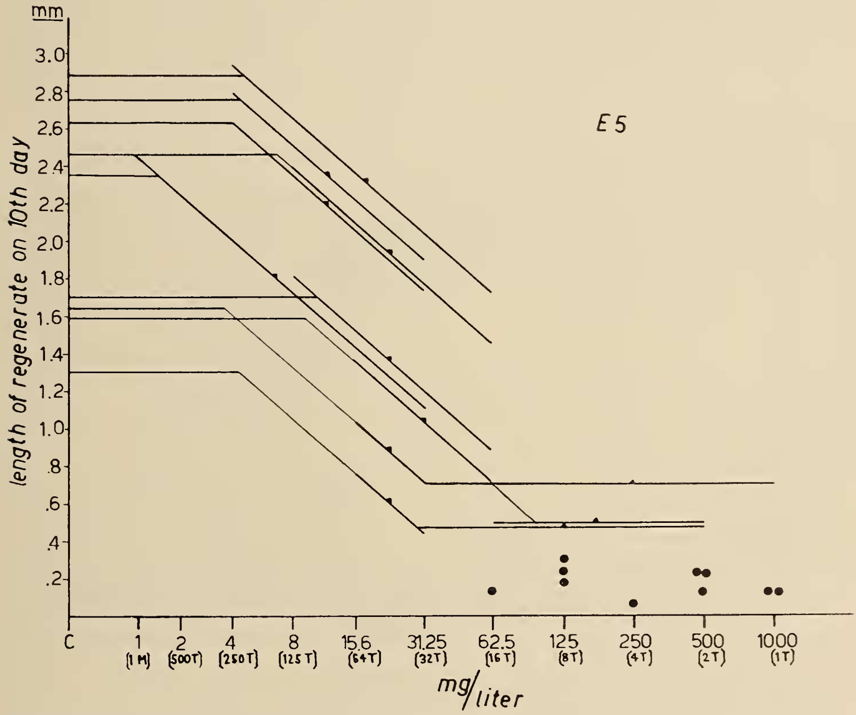


FIG. 4.

Regression lines of 10 day tail regeneration on amino ketone 5 concentration. Each line represents one experiment, five animals per concentration. Slope for the whole group (b) = — 1.056 mm (mean length reduction caused by doubling the concentration (*b* · *log*. 2) = .319 mm).

This same condition and response to re-amputation was also found by LÜSCHER (1946 *a*) in *Xenopus*, and by BERNHARD (1947) in *Rana* after suppression of axial organs by colchicine treatment. Godlewski (1928) also found that after inhibition of tail regeneration in Axolotl by covering the wound surface with a skin flap, growth could be readily induced by re-amputation. These results are in contrast to the permanent loss of regenerative capacity in amphibians after X-raying. (Butler, 1933, p. 281; 1935, p. 305; Scheremetieva and Brunst, 1933, p. 187.) Therefore the chemical inhibition of regeneration is based on a transitory change, while the inhibition caused by irradiation seems to be accompanied by a permanent change in the tissues in amphibians.

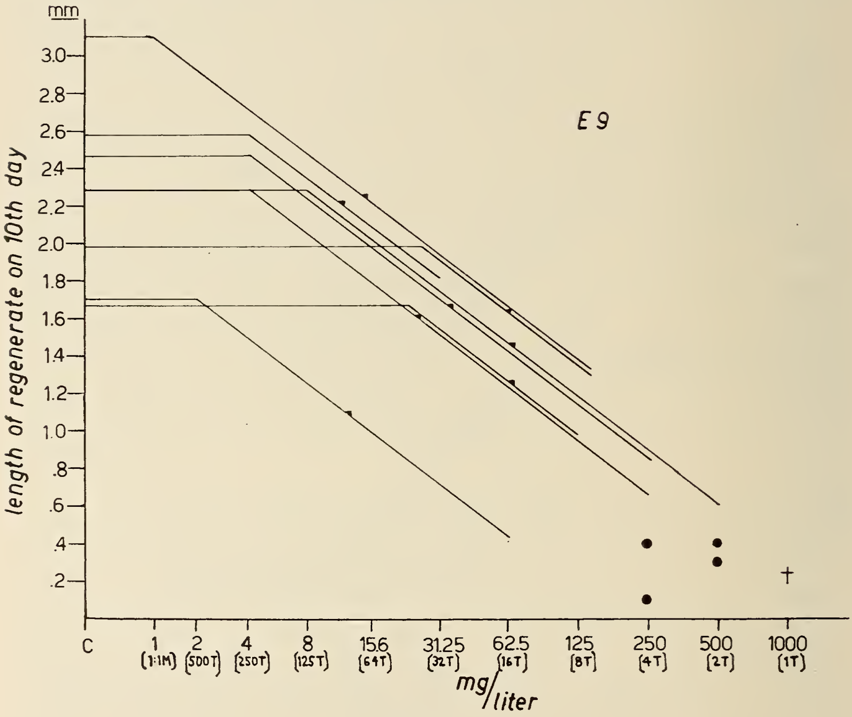


Fig. 5.

Regression lines of 10 day tail regeneration on amino ketone 9 concentration. Each line represents one experiment. Mean slope for the whole group (b) = —.953 mm (mean length reduction caused by doubling the concention (*b* · *log.* 2) = .287 mm).

C. Measureable Effects Upon Growth and Regeneration.

1. *The Degree of Inhibition* (Measurement of length regardless of specific forms of regenerate; see figures 4 and 5).

Up till the present time few substances have been found which inhibit tail regeneration of *Xenopus* larvae to 50% of the control regeneration without having a lethal effect. Only colchicine treatment is able to produce still greater inhibition without lethal effects. Colchicine treatment at 1: 2500 for 30 minutes, for example, produces inhibition of 20-30% of the control regeneration, but is sometimes fatal at this concentration. (Lüscher, 1946 *a*; Lehmann und Bretscher, 1952).

The effectiveness of a given concentration of amino ketone in the inhibition of tail regeneration may be expressed as a percentage of the average control regeneration of the same group. The percentage values are mean values and consequently give no indication of the large variation found in the individual experiments. A statistical evaluation is therefore necessary in order to obtain a true picture of the inhibition.

The concentration-inhibition curve has a more or less exact S. form. To simplify calculations the S curve may be approximated by three straight lines. (Lehmann and Bretscher, 1952.) On the upper level of the curve, which is determined by the mean control regeneration, no effect is found. From a concentration of 1: 250000 to 1: 8000 there is a *progressive increase in inhibition* proportional to change in concentration (descending arm of curve). The regression of tail regeneration on amino ketone concentration was calculated for the descending arm in all experiments (Snedecor, 1948; Linder, 1945). On the lower levels (less than 1.0 mm) inhibition is maximal, constant, and independent of concentration change and is set by animals of the lowest realization grades. The slope of the regression lines proved to be characteristic and statistically constant for each of the substances; ketone 5 = 1.056 and ketone 9 = 0.953 mm. The regression lines are therefore drawn parallel. Figures 4 and 5 show the regression lines for the experiments done with ketone 5 and 9. Figure 6 is an explanatory diagram showing the relationships of the parts of the regression of tail regeneration on concentration.

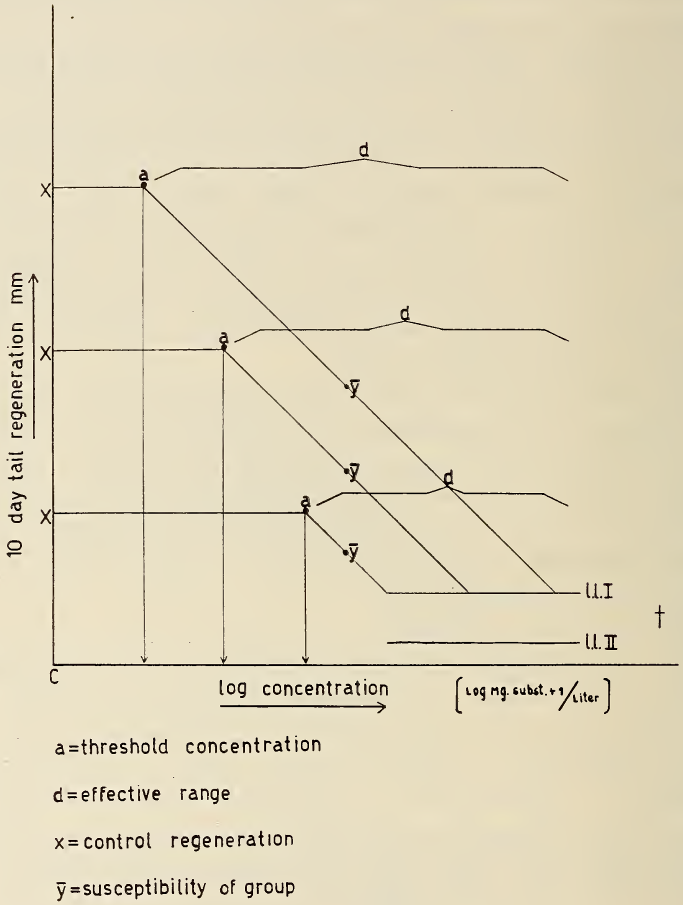


Fig. 6.

Schematic representation of regression lines of tail regeneration on amino ketone concentration showing relationships of the parts. Each line represents one experiment. The slope is statistically constant and the lines are therefore drawn parallel. ll. I and ll. II = lower level I and II. Slope here is statistically = 0 (independent of concentration). Level of 1st horizontal arm is determined by control regeneration length and threshold taken as the point of interception of this arm with the descending arm. Note that, as X (the regenerative intensity) becomes greater: *y* (group susceptibility) increases, *d* (effective range) increases, *a* (threshold) decreases, the slope does not change.

The *maximum degree of inhibition* produced by the amino ketones *at 1: 8000* concentration after 10 days is usually *not greater than 50%* of the average control regeneration. Degrees of inhibition greater than this are always accompanied by suppression of axial organ regeneration.

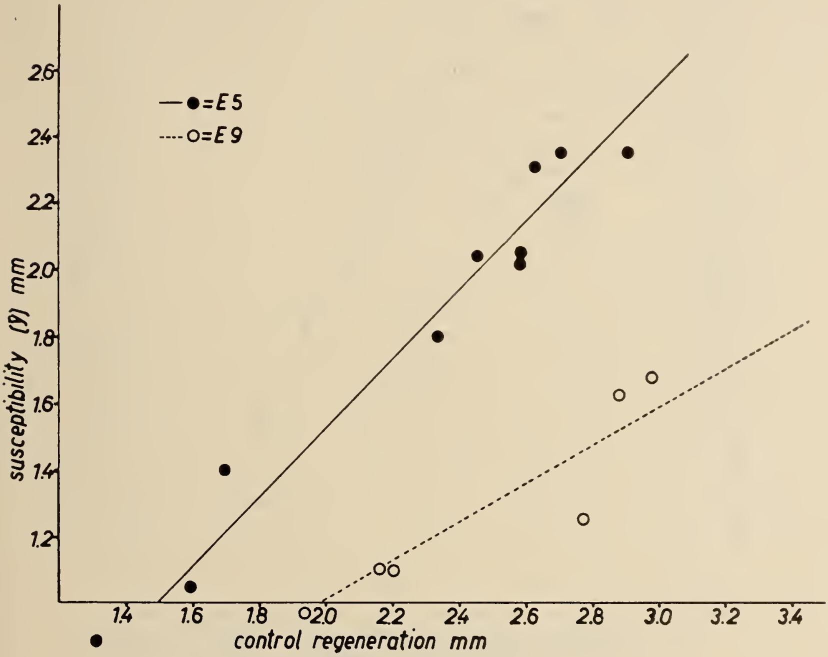


FIG. 7.

Relationship of regeneration intensity to group susceptibility.

2. *Group susceptibility and group Threshold* (see fig. 6).

Group susceptibility ($\bar{y}$) may be here defined as the mean inhibition produced by treatment in one experiment (regeneration on descending arm/number of animals, see fig. 6, and SNEDECOR, p. 103 et seq.). It is a characteristic of each group and may be expressed as a length in millimeters or as a percentage value of control regeneration. Results of several experiments have shown susceptibility to be directly related to the control regeneration of a group (regeneration intensity [1]). This relationship is shown in figure 7, where regeneration intensity, as shown by the control regeneration, is plotted against average susceptibility of the group. The relationship is quite direct but slightly different for amino ketone 5 and amino ketone 9.

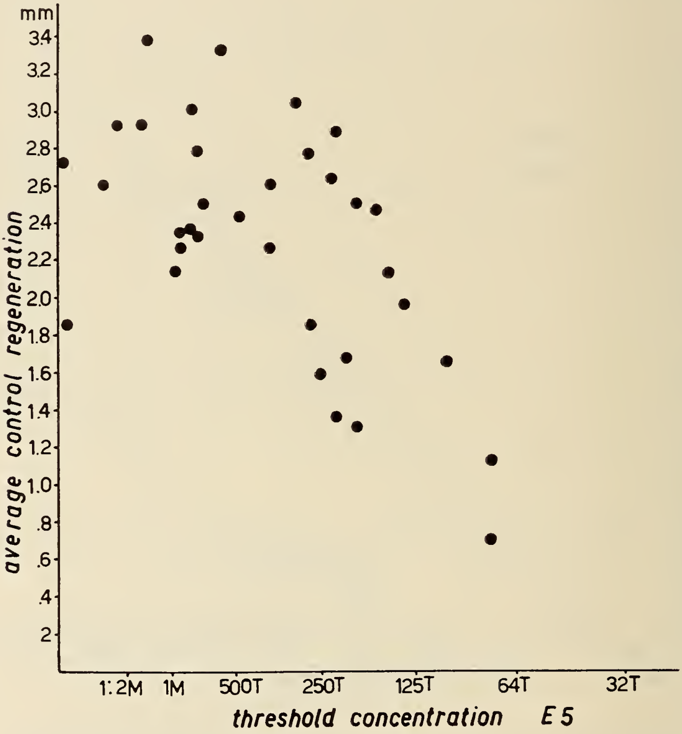


FIG. 8.
Relationship of threshold to control regeneration for ketone 5.

The *threshold concentration* is the most dilute solution able to cause a significant inhibition of regeneration. It varies from experiment to experiment from 1: 1000000 down to 1: 32000 and so it is not possible to give any absolute concentration as a threshold. Because of usual high variability and wide distribution of the individual tadpoles in a group, the threshold concentration was

---

[1] Credit and thanks are here given to Dr. A. BRETSCHER for the methods and terminology used in the statistical analysis of *Xenopus* tail regeneration (LEHMANN and BRETSCHER, 1952).

determined graphically as the point at which the regression line intercepts the horizontal plateau of the mean control regeneration (see fig. 6). When approximated in this manner the threshold shows a relationship to the regeneration intensity of the group and becomes less as the regenerative intensity increases. Figures 8 and 9 show the threshold plotted against control regeneration for amino ketone 5 and 9. Again a small difference is found for ketone 5 and ketone 9.

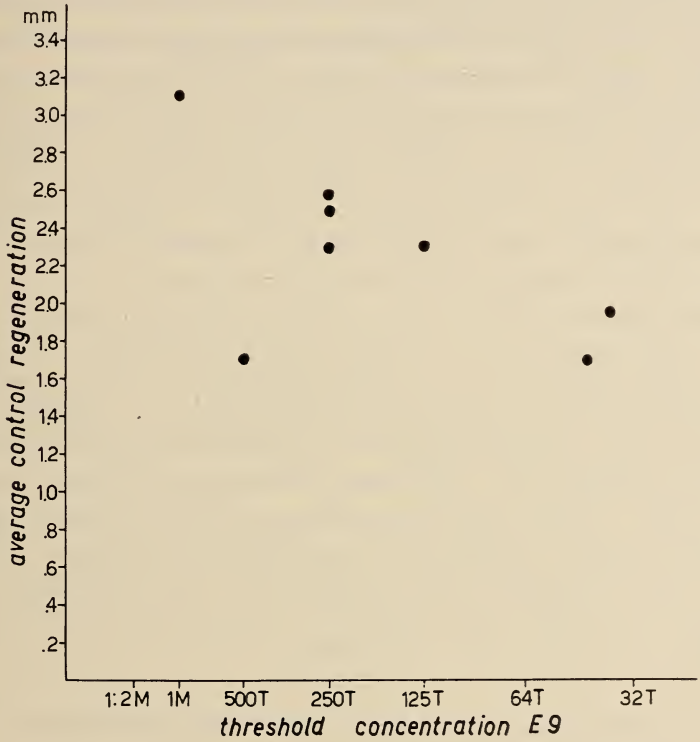


FIG. 9.
Relationship of threshold to control regeneration for ketone 9.

The lower threshold in animals that regenerate more is to be expected on the basis of their higher growth rate and higher metabolic rate which might make them more sensitive to the amino ketones (or their reaction products) than tadpoles with a lower metabolic functional condition. As LEHMANN has pointed out (1947, p. 224), tissues such as blastemas and tumors in which relatively high numbers of cells are ready for mitosis, show a special metabolic condition presumably typified by a high protein metabolism, increase in nucleic acid content, etc. [1]. The biochemically different nature of such tissues increases their sensitivity to some chemical substances and probably is the basis for the selective action of several antimitotic or histostatic substances upon actively growing tissues. Friedenwald (1951, p. 1437) has shown that actively proliferating tissues such as bone marrow, lymph nodes, regenerating liver, etc., have the highest susceptibility to nitrogen mustards and are affected by concentrations that do not affect most other organs. He attributes this high susceptibility of proliferating tissues to the presence of great numbers of pre-mitotic cells which he believes to be especially sensitive.

Gillette and Bodenstein (1946) also found proliferating embryonic tissues to have a much greater susceptibility to nitrogen mustard treatment than non-proliferating tissues. It is reasonable to assume that the sensitivity of regenerating and proliferating tissues to some growth inhibitors increases as the degree of activity becomes more intense. This would be the case with *Xenopus* larvae which show a high regeneration intensity and concomitant higher sensitivity (lower threshold) to amino ketone treatment. Tadpoles which regenerate less intensely are correspondingly less sensitive and higher concentrations of amino ketones are needed to produce an inhibition of regeneration.

3. *Sensitivity as a Function of the Time of Treatment.* (Phase Specificity.)

During the course of the experiments the question arose as to *when* the amino ketones exerted their main effects. Parallel series were carried out in which half of the solutions were renewed on the fifth day while the other half were left for the whole period of ten days. No significant differences were found in the regeneration as a result of this difference in treatment. Since some of the inhibiting substance is taken up by the tissues of the tadpole during the first five days of the experiment, there is less present on the fifth day than on the first. Renewing the amount of substance present by placing the tadpoles in fresh solutions on the fifth day does not however increase the amount of inhibition. A second experiment was undertaken in which ketone 9 treatment was interrupted after one, two, three, four and five days. The results are as follows: Mean control regeneration = 27.5 mm = 100%, 24 hour treatment = 85.9% of control, 48 hour treatment = 62.4%, 72 hour treatment = 47.2%, 96 hour treatment = 44.3%, 120 hour treatment = 45.8%, 10 day treatment = 36.0%. This confirmed the supposition that the main effects may be attained by a treatment of only *three* days. Longer treatment up to five days causes very little increase in the degree of inhibition of regeneration and definitely establishes *the first three days after amputation as the most critical and sensitive phase.* It is of interest to note that the first three days of regeneration (Phase 1) is a time during which blastema formation is taking place by cell migration only. No cell divisions are found in the tail tip during this period. As Lüscher has shown (1946 *b*, p. 722-725), colchicine does not affect Phase 1 of the regeneration process but acts decisively during Phase 2 and 3 when many mitoses are to be found. *The amino ketones are* also *phase specific* in their action, but differ sharply from colchicine in that they exert their main effects *at a time of cell migration* and seem to have little direct effect upon cell division (Lehmann and Dettelbach, 1952).

---

[1] See also: The Chemistry and Physiology of Growth, ed. A. K. Parpart, Princeton Univ. Press, 1949; J. Needham, Biochemistry and Morphogenesis, Camb. Univ. Press 1950, Section 2.72, pp. 442 et seq.; J. Brachet, Embryologie chimique, Masson & Cie., Paris, 1947, Chapitre XI, pp. 444-457.

As has been pointed out in the previous section (C. 2), the biochemically different character of blastemas and proliferating tissues in general is probably responsible for their raised sensitivity to some histostatic and antimitotic substances. The process of regeneration however, like most processes of growth, is not uniform and proceeds by phases or stages, each with a characteristic biochemical nature[1]. It may be assumed that the nucleus and the cytoplasm both take part in the characteristic biochemical " mitotic functional condition " (Lehmann, 1951). Different chemical substances (antimitotics, growth inhibitors, etc.) would therefore not be expected to act upon the same phase of regeneration or to affect all stages equally (Lehmann, *ibid.*). As will be shown (Section III D), the phase specificity of the amino ketones or their reaction products may be clearly demonstrated upon histological examination of treated tissues which have been fixed after various times of treatment. *

---

[1] As examples the following may be noted: Okuneff (1928, 1929, 1933) studied pH, buffering power, and lactic acid content in regenerating tissues and found that all were decidedly different from normal tissues and varied in amount during the course of regeneration. Ryvkina (1940) has shown that during the earliest phases of regeneration reduced glutathione increases in amount while oxidized glutathione decreases steadily. During later stages of regeneration oxidized glutathione again increases.

Ghiretti (1950) has found that acid and alkaline Phosphatases show maximum activity in regenerating tail tissue at the time of blastema determination on about the 10th day... (p. 19).

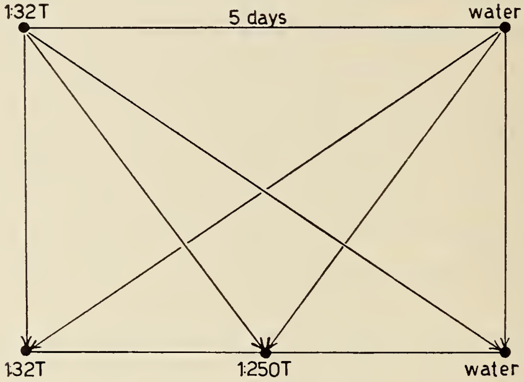


Fig. 10.

Diagram of the " pre-treatment " experiment. Each arrow represents 10 animals. Amputation was carried out after 5 days of pre-treatment. The experiment was also carried out once with half as many animals.

4. *Pre-treatment.*

In order to determine if the inhibitor substances are taken up from the solutions other than through the wound surface only, and to determine if they can still influence regeneration when so applied, the following experiments were performed.

Thirty tadpoles of the same size were maintained together in 900 cc of 1:32000 solution in a 1.5 liter cylindrical aquarium for five days. Thirty other tadpoles of the same length and from the same egg group were kept in 900 cc of distilled water in a like container. After five days of this pre-treatment, 4 mm was amputated from the tail tip of each of the animals. After amputation, each group of thirty animals was divided at random into three smaller groups of ten each and the groups distributed 5 per 150 cc glass into 1: 32000, 1: 250000, and distilled water. This was done for both the animals that had been kept in the amino ketone solution before amputation and for those that had been in water. For clarity the experiment is diagrammed above. (Figure 10.)

---

* See also F. E. Lehmann, Einführung in die Physiologische Embryologie, Verlag Birkhäuser, Basel, 1946, for a discussion of phase specifity of embryonic anlagen.

The results of the experiments are given in the table below.

TABLE 1. — *Results of the pre-treatment experiments. In every case and at all concentrations pre-treatment caused a significant difference.*

Pre-treatment		1: 32000				Water			
Post-treatment . . .		1: 16T	1: 32T	1: 250T	H_2O	1: 16T	1: 32T	1: 250T	H_2O
% of Control Regeneration	Exp. 1	47.6		75.5	73.5	58.0		91.0	100.00
	Exp. 2		70.5	84.0	92.5		82.3	93.5	100.00

Statistically there is a significant concentration difference (as expected), a significant pre-treatment versus non pre-treatment difference and a significant interaction difference.

The pre-treatment causes a reduction of the regeneration intensity which is measurable on the controls (figure 11 and 12). The mean regeneration at each concentration is accordingly less for all of the pre-treated animals. The slope of the concentration-regeneration curve, however, is not changed by pre-treatment and the only effect on the group is a lowering of the regeneration intensity without an increase in sensitivity as shown by the unchanged slope. In this sense there is *no cumulative effect.*

The possibility remained however that the pre-treatment had effected a difference in the total size of the animals prior to amputation great enough to account for the differences found ten days after amputation. Twenty tadpoles were therefore divided into two groups and subjected to ten days of pre-treatment in order to

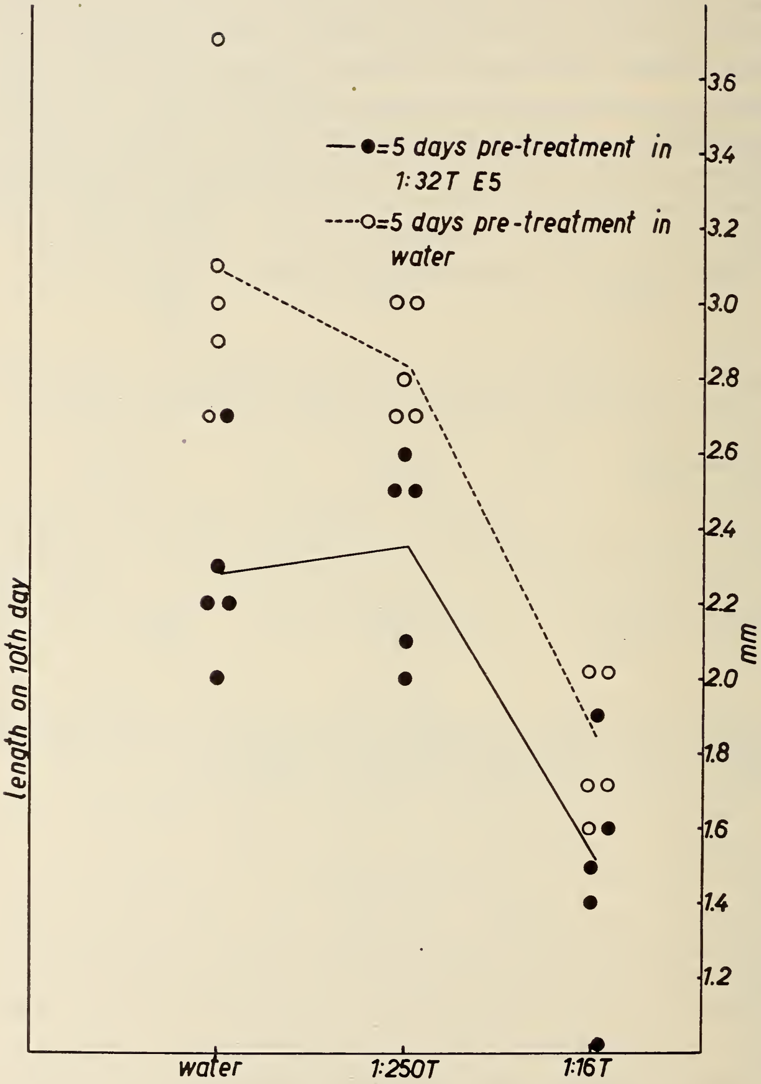


Fig. 11.
Effect of 5 day pre-treatment at 1: 32000 on tail regeneration at different concentrations.

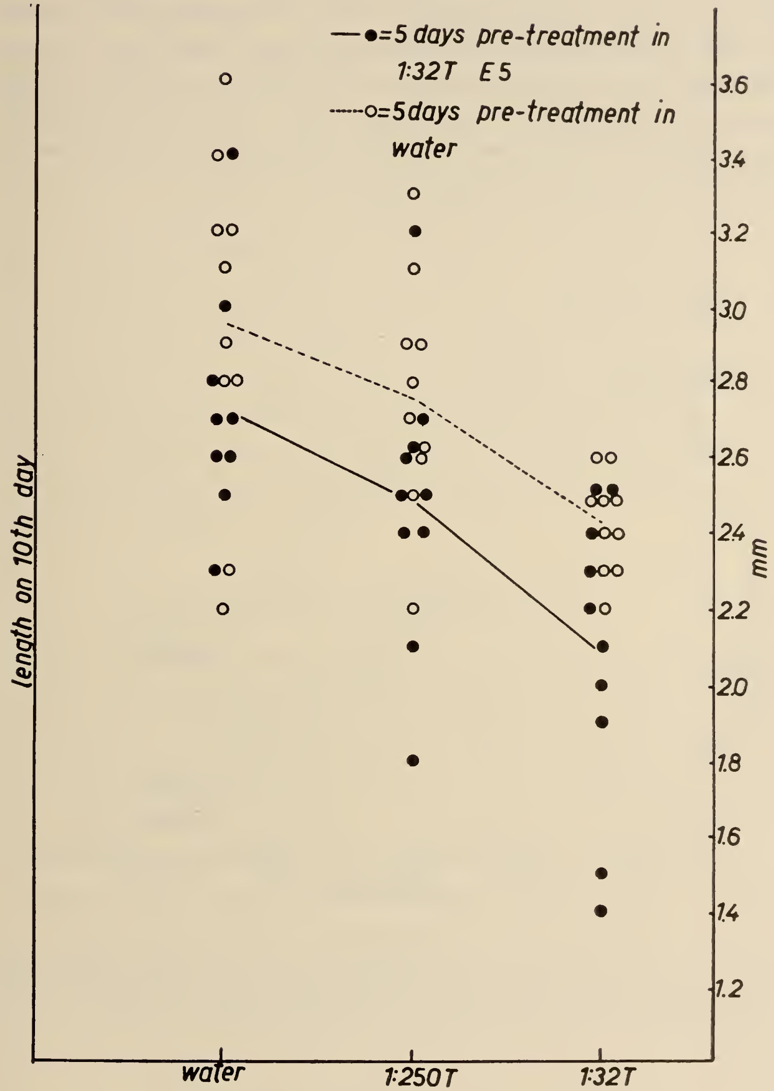


FIG. 12.
Effect of 5 day pre-treatment at 1: 32000 on tail regeneration at different concentrations.

determine if a size difference resulted at the end of five days of 1: 32000 treatment. There was a significant difference in the total length from the control length (P = 4%). Pre-treatment caused a reduction in body length of about 8% after 5 and after 10 days which itself is statistically significant but which is not great enough to significantly influence tail regeneration. Therefore the inhibition of regeneration caused by pre-treatment at 1: 32000 for five days may be accepted as significant.

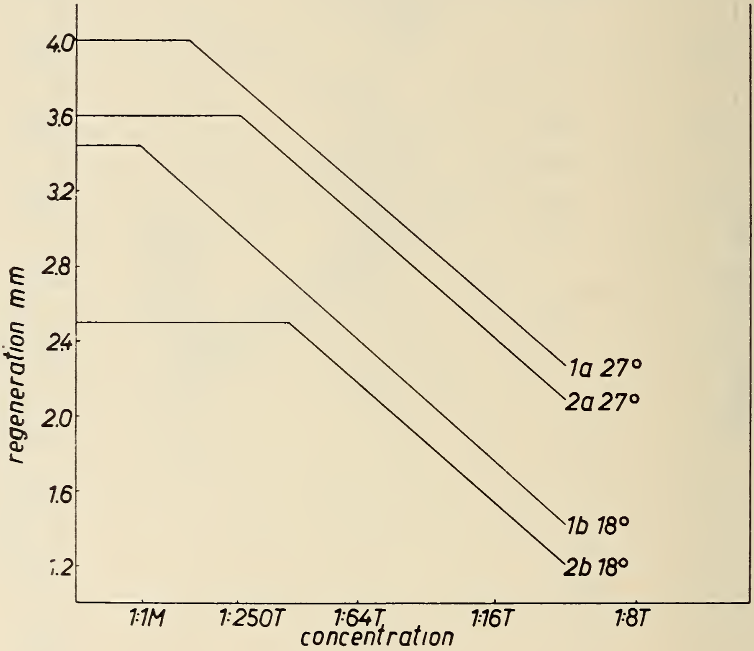


FIG. 13.

Regression lines of 10 day tail regeneration on ketone 5 concentration; effect of temperature variation. 1a 27° C, 1 *b* 18° C. 2 *a* 27° C (6th day), 2 *b* 18° C. Slope remains the same, regeneration intensity raised at 27° C.

5. *Effect of Temperature Variation.*

In order to determine if temperature plays a part in the efficacy of the amino ketones upon regeneration, experiments were conducted with animals from the same egg batch and of the same size in which half were maintained at 27° C and half at 18° C. The usual range of concentrations (1: 8000 to 1: 1000000) plus 10 controls at each temperature were used. Although an increase in temperature speeds the process of regeneration considerably, no significant difference was found in the amount of inhibition at the two temperatures used. The relative amount of inhibition remains the same (statistically) at the various concentrations at the higher temperature. (The slope of the regression lines remains unchanged.) Due to the acceleration of the growth rate at the higher temperature, the tail regeneration is much farther advanced after 5 and after 10 days at 27° C than it is at 18° C. Important however is not the absolute length of regeneration but the relationship of the treated animals to the controls of the same experiment. In order to be able to compare more directly the results of the temperature experiments, measurements of the 27° C animals were made on the 3rd and 6th days and compared with the usual measurements on the 5th and 10th days of the 18° C animals. The regression lines are shown in figure 13.

6. *Toxicity and Effective Range.*

The amino ketones show a relatively low toxicity. The larvae are able to withstand concentrations as high as 1: 2000 (500 mg per liter) and 1: 1000 (1000 mg per liter) for ten days without succumbing. At a concentration of 1: 8000 or weaker there were never any lethal effects found. This is a remarkably low toxicity when compared for example with colchicine. *Xenopus* larvae treated with colchicine for only 30 minutes at a concentration of 1: 1000 or 1: 2000 show 100% mortality (Lüscher, 1946 *a*, p. 483). The lethal effects of the amino ketones at the stronger concentrations seem practically independent of the size or age of the tadpoles. There is a slight tendency for animals of 25 to 30 mm to withstand treatment at higher concentrations without succumbing. Larvae of this length are in a very active growth phase and nearing the stage in which the hind legs first appear. This high metabolic condition may account for the higher resistance (as well as the greater sensitivity as already noted).

Due to the low toxicity and the rather gradual slope of the concentration-inhibition curve, the amino ketones possess a large effective range. The effective range varies from batch to batch and is dependent upon the regeneration intensity of each group. The range of concentrations that are effective (descending arm of concentration-inhibition curve) is greater as the threshold concentration (Fig. 8 and 9) becomes less, which in turn decreases as the regenerative capacity increases. In other words, *the greater the intensity of regeneration of the tail, the greater its sensitivity to treatment and hence the greater the range over which regeneration is affected.* For example in the ketone 9 experiments, one series had a mean control regeneration of only 17.0 mm while the threshold was at 1: 64000. In a second series mean control regeneration was 23.0 mm and the threshold 1: 125000, a third series had a mean control regeneration of 31.0 mm and a threshold concentration of 1: 1000000. Because of the continually decreasing threshold with increase of regeneration intensity the effective range also becomes greater (lethal concentration practically constant).

D. HISTOSTATIC AND CYTOSTATIC EFFECTS UPON THE REGENERATING TISSUES.

(See also LEHMANN and DETTELBACH, 1952.)

The effects of the amino ketones upon the measurable inhibition of regeneration give only some quantitative data but little information on the mode of action of the inhibiting substances. For this purpose a systematic histological and cytological examination was undertaken on a material which was composed of more than ninety tadpole tails treated at 1: 16000 and fixed after 12, 24, 36, 48, 72, 96 and 120 hours plus controls for each period. Two series each of ketone 5 and ketone 9 treated animals with controls were sectioned. Since LÜSCHER (1946 *b*), NAVILLE (1924), IDE-ROZAS (1936), HELLMICH (1929) and others have already given a description of the normal histology of regeneration, the description given here is limited to the effects caused by treatment with the amino ketones. The results of this study, in conjunction with the other results, give some insight into the mechanism of action of the inhibiting substances upon the process of tail regeneration and show the parts which are most severely affected by treatment.

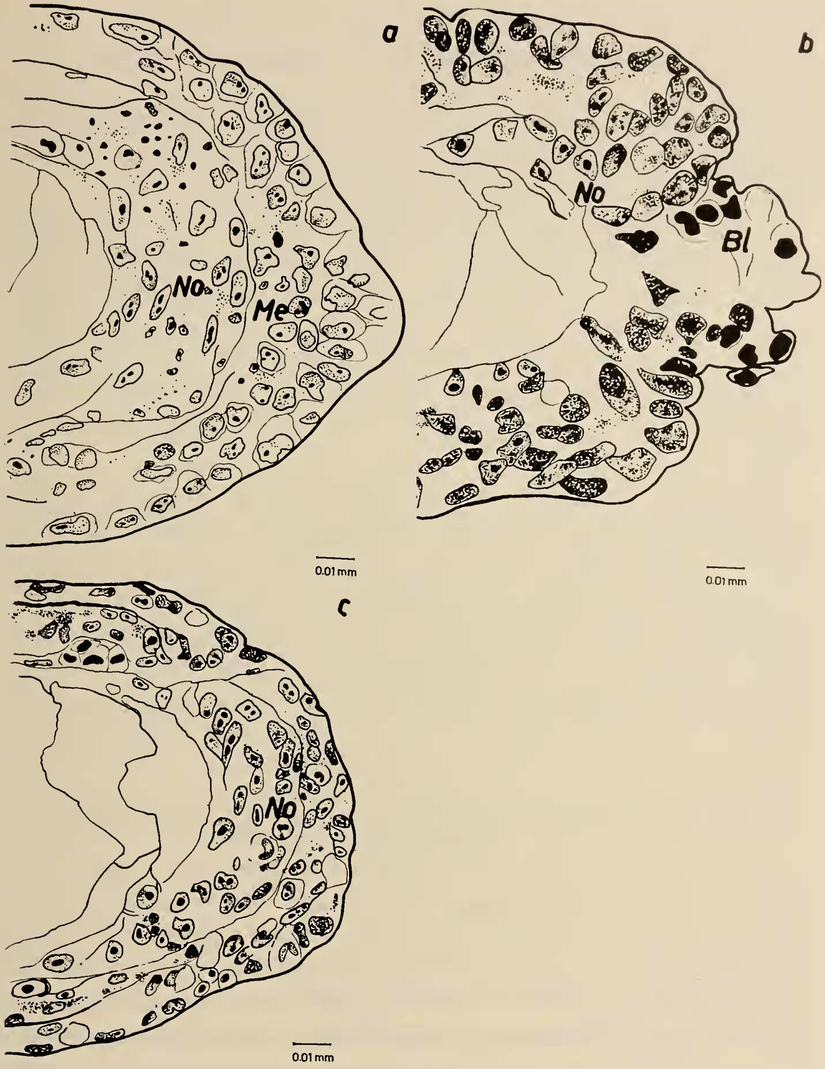


Fig. 14.

Camera lucida drawings of sagittal sections of tail tips fixed 36 hours after amputation. *a* = control, *b* = treated with ketone 9 at 1 : 16000 ; *c* = treated with ketone 5 at 1 : 16000. No = notochord, Me = mesenchyme area, Bm = basal membrane, Bl = blood cells.

1. *Epidermis* (Inhibition of the activation process).

After amputation of the tail tip, some blood is lost before a clot is formed. By 36 hours the tip is covered with epidermis which has closed over the wound surface and pushed the clot inside.

In the 36 hour control the epidermis may be seen to be quite regular and somewhat thickened at the immediate tip (Fig. 14 *a*). A short distance proximally the epidermis becomes a layer two cells deep with flattend nuclei. At the tip the nuclei are large with prominent nucleoli. Most cell boundaries are sharp and the epidermis is clearly delimited from the adjoining tissues. The normal epidermis appears in this phase as a strongly activated tissue.

As a result of treatment, organization of the epidermis at the tip is much impeded and the formation of a two celled layer much delayed. The effects are very clear after 36 hours when the nuclei become misshapen, and a deficiency in the number of nucleoli is apparent. This general picture varies according to the concentration applied. It is also somewhat more the case after treatment with ketone 9 than it is after ketone 5 treatment (Figs. 14 *b* and 14 *c*). Cell boundaries are often not sharp and the tip epidermis is not clearly delimited from the underlying tissues. There is also a considerable amount of granulation to be seen and many cells show vacuoles and signs of pyknosis. The epidermal covering of the tip is often found to be thickened at the early stages after amputation (up to 48 hours) with many closely packed nuclei which give a very dense appearance to the tissue. The above mentioned effects are very evident after treatment at 1: 16000 till abour 72-96 hours, at which time they begin to disappear (Figs. 15 and 16).[1] By 120 hours after amputation the inhibited epidermis has recovered extensively and appears about like that of the 48 hour controls. The inhibited state of the epidermis after treatment is clearly shown by all the changes noted above.

---

[1] Figures 14 to 16 are *camera lucida* drawings of sagittal sections of control and treated tails which have been fixed 36, 48 and 72 hours after amputation.

2. *Basal Membrane* (Inhibition of transformation).

The basal membrane lies immediately under the epidermis covering of the tail and delimits this layer from the adjoining tissues. In the 36 hour control it extends as far distally as the base of the notochord cap, which point also marks the level of amputation. It remains in about this relationship to the notochord during the whole period of active growth and keeps pace with the notochord sheath in most cases. As a result of amino ketone treatment a difference is found, especially at 36 and 48 hours, when the basal membrane extends beyond the notochord cap distally (Figs. 14 and 15). It seems very improbable to attribute this condition to an acceleration of growth of the membrane in view of the retardation of most of the other tissues. In the normal course of events following amputation, the basal membrane receeds slightly (probably it undergoes histolysis; see the next section) and then starts its regeneration after the notochord cap has been formed. After treatment this process is evidently disturbed and the basal membrane appears to project beyond the cap of the notocord. At 72 hours and more after amputation the basal membrane takes on a more or less normal position in the regenerating tail, and unless treatment has been very severe no further abnormalities are to be seen.

3. *Mesenchyme* (Inhibited organization of mesenchyme space. Delayed histolysis).

The area between the notochord cap and the tip epidermis (Figs. 14 *a*, 15 *a*, 16 *a*) presents a rather loose appearance in the controls of 36 hours. Many nuclei are to be seen and this area is quite distinct from the adjoining tissues. This condition prevails as regeneration proceeds and the area elongates as the regenerate grows.

In tadpoles treated with amino ketones the formation of this area is considerably disturbed. In its place is found a compact mass containing cellular debris, blood cells, and which is similar to that of the controls shortly after amputation (Figs. 14, 15 and 16). Mesenchyme cells which are always to be seen in the controls of 36 hours are absent in treated tails of the same age. After treat-

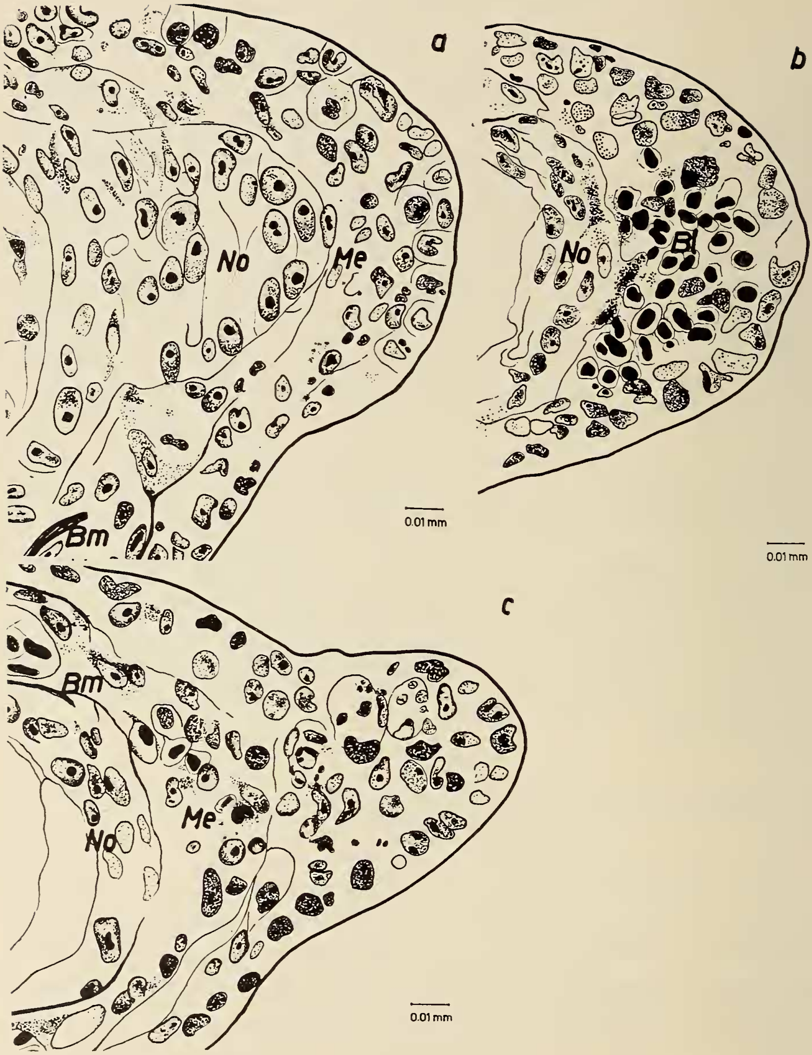


Fig. 15.

Camera lucida drawings of sagittal sections of tail tips fixed 48 hours after amputation. a = control, b = treated with ketone 9 at 1: 16000; c = treated with ketone 5 at 1: 16000. Abbreviations as in Fig. 14.

ment at 1: 16000 the effects are evident in this area till about 96 hours, after which the condition begins to improve rapidly.

The formation of a loose and somewhat de-differentiated area at the space of active growth of the notochord seems essential for the proper regeneration of the tail. Most probably proteolytic enzymes (autolytic enzymes) play the chief role in the opening of this area and the removal of the blood clot left here after amputation. HELLMICH (1929), POLEŽAJEW (1933) and JEFFIMOFF (1933) have all suggested that one of the main functions of the epidermis is a histolytic one, the result being a removal of debris and a lessening of physical resistance to outward growth. POLEŽAJEW and FAWORINA (1935) after experiments on limb regeneration in *Axolotl* postulate that the role of the epidermis immediately after amputation is largely a histolytic one and through this process there is an apparent freeing of cellular material which later contributes to the regeneration blastema. In the regenerating *Xenopus larva* tail the equivalent process is probably the formation of free mesenchyme cells in the area beneath the tip epidermis (although they play a very minor role in histogenesis of tail regeneration). BROMLEY and ORECHOWITSCH (1934) studied tail regeneration in larvae of *Pelobates fuscus* and *Axolotl* and found a very great increase of cathepsin in the stump and blastema tissues during the first three days after amputation. RYVKINA and STRIGANOVA (1939 *a*, 1939 *b*) found an increase in cathepsin during mammalian wound healing, and ADOVA and FELDT (1939) have shown in *Axolotl* that those parts of the epidermis that will support regeneration are richer in some kinds of digestive enzymatic activity than epidermis from other parts of the body (see also NEEDHAM, *op. cit.*, pp. 444 et seq. and STRIGANOVA, 1940 *a* and *b*).

KAUFMANN and NEURATH (1949) have described an inhibition of α-chymotrypsin by means of 1-phenyl-2-acetaminobutanone-3 acting as a competitive substrate. It might be possible that the amino ketones (actually, amino hexanones) or their reaction products act in some similar way to inhibit the tissue cathepsins which have been activated by the increase in lactic acid content and fall of pH in the regenerating tissues. (OKUNEFF 1928 and 1933.) (See also SUMNER and SOMERS, p. 192). Due to the failure of the epidermis to carry out its normal functions and the disturbance of the histolysis of the tissues, the area beneath the tip epidermis becomes a possible physical barrier to the outward growth of the axial organs. The resulting alteration of the chemical environment at the tail tip undoubtedly also participates in retarding the regeneration process. *It is possible then, that the amino ketones exert a strong effect on regeneration by inhibiting or suppressing in some way the formation or activation of the proteolytic enzymes necessary for the beginning of normal regeneration.*

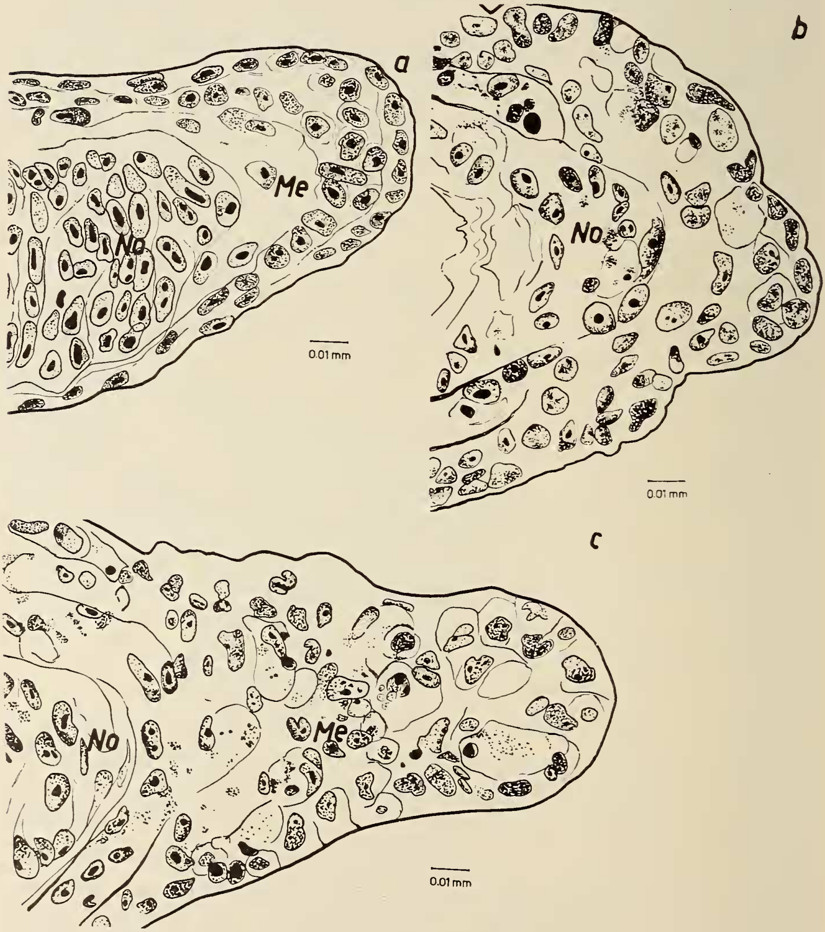


Fig. 16.

Camera lucida drawings of sagittal sections of tail tips fixed 72 hours after amputation. a = control, b = treated with ketone 9 at 1: 16000; c = treated with ketone 5 at 1: 16000. Abbreviations as in Fig. 14.

4. *Notochord — Blastema and Sheath* (Inhibited organization and reduction in size).

As already mentioned (Section III, 3), the notochord plays a very important role in tail regeneration. It is therefore of great interest to know how it is affected by amino ketone application. The notochord usually projects beyond the wound surface during the first 24 hours after amputation and is entirely covered by its sheath during this part of phase 1. By 36 hours the notochord cap blastema is composed of cells with large oval nuclei, most of which possess two clear nucleoli (Fig. 15 *a*). The nuclei are oriented with their long axes perpendicular to the long axis of the tail. The normal regenerating notochord cap has a typical blastematic appearance and develops a cone form as growth progresses. New cells form distally while vacuolization takes place at the base of the cap.

Treated animals show a notochord cap that is much less distinct, with smaller nuclei, small and often absent nucleoli and a high degree of aberration from the normal form. Amino ketone 9 produces these effects more strongly than does ketone 5, and in the 36 hour treated animal there is a small notochord cap present after ketone 5 application but after ketone 9 the cap is represented by only a very few cells that are poorly organized (Figs. 14 and 15). *The poor organization of the cells of the notochord blastema (and the epidermis) is a very characteristic effect of the amino ketones and is usually accompanied by a reduction in cell size as well as number* (see part 7). In later stages (phase 2 and 3), and especially if the treatment has not been too severe, the notochord cap cells begin to approach the normal in appearance. Five days after amputation the cap is still too small but the cells show a good orientation and resemble the controls of about the third day. The reduced size of the blastema is probably responsible for the reduced length of the regenerating notochord (lower realization grades, see p. 349).

In the controls the notochord sheath consists of a single layer of flattened cells that enclose the notochord at all places except at the very tip. The sheath shows no definite abnormalities after treatment and the only perceptible variation from the controls is its retarded growth. The appearance that the notochord sheath encloses more of the notochord cap in the treated animals is probably due to the inhibition of the outward growth of the notochord itself and not due to any changes in the sheath. The disturbance of the tissue proteolytic enzymes may also have some effect on delaying the growth of the sheath.

5. *Spinal cord* (No reaction).

Only very slight signs of the effects of treatment may be seen in the spinal cord. Small differences are found in the affinity of the treated tissue for stains and the nuclei appear a bit more compact than the controls. Other than a retardation of growth the neural cord seems hardly affected. The effect upon growth mentioned is probably due to the suppression of the growth of the notochord and seems not to be a direct result of treatment with the amino ketones.

6. *Mitoses* (Delayed but normal mitoses).

The onset of mitoses in the stump is delayed after treatment. At 72 hours after amputation the controls show the initiation of mitotic activity in the stump tissues while in the treated animals none are to be seen at this time. At 96 hours in the controls mitoses are to be found in great numbers in the stump and also in the new tissues as well. At 96 hours in the experimental animals some mitoses are to be seen but with a much lower frequency than in the controls. This delay is more pronounced after ketone 9 than after ketone 5 treatment. The numbers of mitotic figures increases from this time on and after the fifth day begins to approach the control frequency. *The mitoses in the treated animals appear quite normal and all stages are found in the normal ratios.* It seems that once the cells begin to divide, mitosis runs its normal course. This action is in sharp contrast to colchicine which acts upon the nucleus of cells entering mitosis causing a block at metaphase and usually death of the cell. The amino ketones apparently do not exert their main action upon cell division although the numbers of dividing cells are reduced. It is possible however that the amino ketones affect one or more of the numerous intra-cellular enzymes known to be present in the cell organoids (see BOURNE 1951, Chap. VI, and ROBERTIS, NOWINSKI, SAEZ 1948, Chap. V) without causing mitotic abnormalities. This could cause a lowered cell vitality and would in part account for the *lower frequency of dividing cells in the early phases of regeneration.*

7. *Nuclear Size Variations* (Reduction of size and number).

Upon comparison of the treated fixed tails with the controls there appeared to be a difference in the size of the nuclei in the notochord cap and in the epidermis. An indication of the significance of nuclear size in nonpolyploid cells is given by the following: HINTZSCHE (1949) has found that the nuclei of functional cells of the human uterine epithelium show an increase of volume during the regeneration period and a decrease in volume as the end of the cycle is neared. He found a similar relationship between nuclear volume and the functional condition in the human ovary (1945) as well as for intestinal epithelium (see also JACOBJ, 1941). RISLER (1950) has reported changes in nuclear volume which parallel tissue growth in an insect larva. STICH (1951) has shown that the nuclear size in *Acetabularia mediterranea* is reduced in the absence of light (reduction of metabolism) and increases sharply when light is admitted (activation of metabolism) or during regeneration. In the normal regenerating *Xenopus* tail the notochord blastema nuclei are very large and stain intensively red with a methyl green-pyronin mixture (in controls treated with ribonuclease the sections stain only green). (See BRACHET, 1950.) This indicates the presence of large amounts of ribonucleic acid and a high rate of protein synthesis. (CASPERSSON, 1941, 1947; BRACHET, *op. cit.*; CLÉMENT-NOËL, 1944). It seems then, that an increase in nuclear size is closely correlated to high functional condition in many tissues. Whether the contrary is true is more difficult to say at present, but a failure of nuclei to attain a size shown by the controls probably is connected with a reduction of the synthetic capacity of the cells. Inhibition of nuclear growth may also be responsible for a reduction of cell division rate (directly or indirectly), thereby causing an inhibition of growth.

In order to determine if the difference in nuclear size is a real one and to be able to compare the various nuclei, drawings were made with the *camera lucida* at 1700 × magnification and the nuclear outlines thus obtained planimetrically measured. Since the effects upon the tissues are most pronounced at early stages and because the most sensitive period is within the first three days, the examination was carried out on tails which had been fixed 36 and 48 hours after amputation and which had been treated at a concentration of 1: 16000.

The results showed quite a large dispersion and it was necessary to compare them statistically to be certain of the differences. The mean values of the notochord and epidermis at 36 and 48 hours were compared by means of the t-distribution (Snedecor, 1946; Linder, 1945). The mean surface areas are given in the table 2 below. As absolute values were not considered important, the values shown are relative and do not indicate the actual dimensions.

Table 2. — *Planimetrically determined surface areas of notochord and epidermal nuclei at 36 and 48 hours after amputation.*

	Control		A. K. 5		A. K. 9	
	Noto.	Epid.	Noto.	Epid.	Noto.	Epid.
36 Hours	28.8	20.8	16.9	14.8	27.3	21.5
48 Hours	34.3	21.9	26.1	18.6	14.2	16.8

The following conclusions may be drawn from these measurements and their statistical evaluation.

Notochord (Figure 17).

1. 36 hours:

At 36 hours after treatment at 1: 16000 with amino ketone 5 the nuclei of the regenerating notochord cap cells are significantly smaller than these of the controls. Similar treatment with ketone 9 does not produce a reduction of nuclear size but the cell number and tissue area is here greatly reduced and the nuclei show the effects to a greater degree than after treatment with ketone 5.

2. 48 hours:

After 48 hours of ketone 5 treatment the notochord cells have increased in size but are still significantly smaller than those of the controls. After the same time of ketone 9 treatment the notochord cap nuclei have become very small but have increased in number slightly. It may be seen here that the two substances affect the regenerating notochord differently with ketone 9 being the stronger but affecting cell size later than ketone 5.

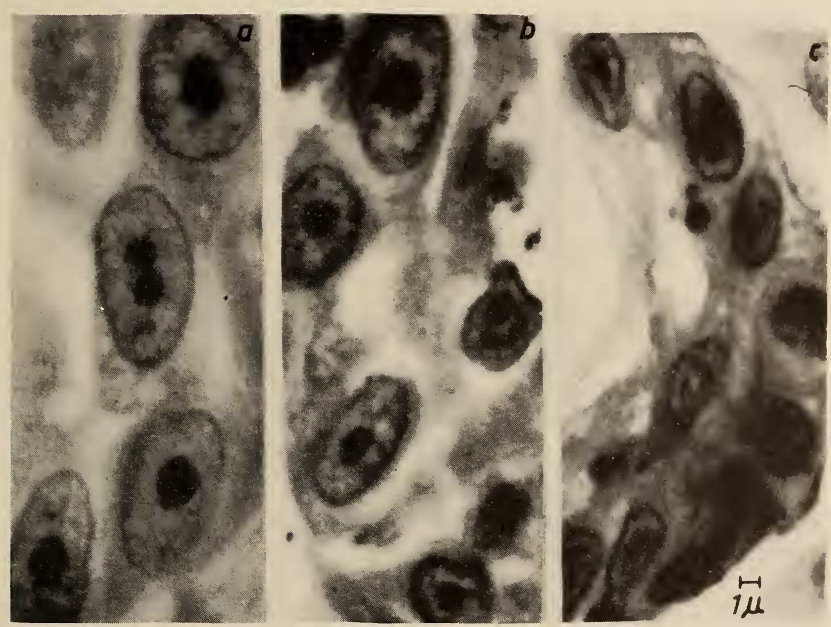


FIG. 17.

Notochord blastema cells fixed 48 hours after amputation. *a* = control, *b* = treated with ketone 5 at 1: 16000, *c* = treated with ketone 9 at 1: 16000. Note the reduction of nuclear and nucleolar size after treatment. In *c* the number of blastema cells is also reduced.

Epidermis:

1. 36 hours:

The nuclear size of the tip epidermis after ketone 5 application is significantly at 36 hours smaller than that of the controls, while ketone 9 produces only a small and *in*significant reduction in nuclear size up to this time.

2. 48 hours:

After ketone 5 for 48 hours the epidermal nuclei show a small increase in size but are still significantly smaller than the controls. In animals treated with ketone 9 the nuclei of the tail tip epidermis show a great reduction in size and not only are smaller than those of the controls but also are smaller than those from ketone 5 treated tails.

Both the notochord and epidermis show improvement in nuclear size from 36 to 48 hours after treatment with amino ketone 5. Not only is the contrary true after ketone 9 treatment, but the amount of tissue regenerated is considerably less and the effects of treatment much more evident. This method of examination confirms and extends the differences already found in the action of the two substances.

8. *Nucleolar Size Variations* (Reduction of size and number)

What has been said at the beginning of this section on the significance of the size of the nucleus applies to the *nucleolus* as well. As already mentioned, *a poor condition, small size, and or absence of the nucleoli in epidermis and notochord after amino ketone treatment is regularly found* (see fig. 17). Caspersson and Schultz (1940), Brachet (1947) and others have pointed out the connections between the nucleolus, nucleic acids, and protein synthesis. Stich *(op. cit.)* found regular cyclic changes of nucleolar volume corresponding to changes in nuclear volume after varying the amount of light (and therefore assimilation) and in regeneration experiments on *Acetabularia.* He concludes that an intensive protein synthesis (as in growth or regeneration) is accompanied by rapid increase in nuclear and nucleolar mass. Yokoyama and Stowell (1951) have reported nucleolar enlargement during phases of rapid synthesis of protein rich zymogen granules in mouse pancreas after stimulation by pilocarpine injections. Stowell (1948) has also shown that the nucleoli of regenerating rat liver increase greatly in volume within 24 hours. Damage to the nucleoli after treatment by the amino ketones therefore suggests interference with nucleoprotein synthesis and metabolism. *The amino ketones or their reaction products act upon the interphasic nucleus, — either* *directly or through the complex cytoplasm surrounding the nucleus* (Lehmann, 1951) — *and by altering the cellular functional condition, reduce the synthetic intensity (or capacity) of the cells and the numbers of cells entering into mitosis.*

IV. THE EFFECTS OF AMINO KETONES UPON EMBRYONIC CHICK FIBROBLASTS CELLS *IN VITRO*

The use of tissue cultures as a biological test material is well known.[1] As in regenerating tissues, cell migration and many mitoses occur in cultures of cells *in vitro* and may be determined quantitatively. It is possible, therefore, to obtain a quantitative expression of the effectiveness of the amino ketones by applying them to tissue cultures.

A. Methods and Materials.[2]

The experiments were performed on tissue cultures grown by the hanging drop method. In most experiments embryonic heart tissue from the chick was used in a medium composed of one drop of plasma, one drop of embryonic tissue extract, and one drop of the test or control fluid in sterile Tyrodes solution. The amino ketones were thus diluted to ⅓ their original strength. The concentrations in the cultures were 1:48T, 1: 24T, 1: 12T, 1: 6T, 1: 3T, 1: 1500 and 1: 750 — three cultures being made at each concentration plus 3 to 8 controls for each experiment (10 in all). Incubation was at 36 to 37° C and after 48 hours the cultures were fixed in Carnoys fluid, stained with hämalaun and mounted in Canada balsam. Surface areas of the explant and the 48 hour growth were projected and planimetrically measured before fixation. An area index was then calculated from these values as: total 48 hour growth area/explant area. Mitotic activity was estimated by making systematic counts of all cells in rows chosen at random that passed closely by the opaque explant and thus included the more dense areas as well as the thinner areas lying more peripheral. The mitotic index was then calculated as: Mitoses/Total cells × 100. Phases of mitosis were also noted during the counts.

---

[1] For extensive literature see, R. C. Parker, 1938, pp. 262-265, Lettré, 1950; Gaillard, 1942.

[2] The cultures were prepared, measured, fixed and stained in the laboratories of the Theodor Kocher Institut, under the direction of Professor E. Hintzsche, to whom I wish to express my thanks.

B. Effects Upon Cell Division and Cell Morphology.

(At strong concentrations rate of division reduced,
vacuoles produced, but mitoses normal.)

The M/C × 100 proved to be very irregular even within the controls of one series of cultures. For example, in one series there was such a wide spread of the values of the mitotic coefficient of the control cultures (0.46 to 1.30) that it would be difficult to consider them as an homogenous group. In the same experiment mitotic coefficients from cultures treated with 1: 12000 amino ketone varied from 0.73 to 2.18 and at 1: 750 from 0.7 to 1.5. This situation was found in all of the experiments made. It appears that as long as there is some growth in a culture, there is a more or less normal ratio of dividing to resting cells, although the total cell number may be considerably reduced in the peripheral areas. This indicates that the amino ketones are not mitotic poisons and substantiates the findings on *Xenopus* larvae although the possibility exists that they may cause a slowing of mitosis as well as a reduction of the number of cells entering mitosis. Because of the non-homogenous nature of the cultures an exact quantitative analysis of the rate of cell division was found impossible.

Mitotic figures seemed normal in treated cultures and all phases were found at all concentrations where growth was not entirely suppressed (Figure 18). There seemed a slight tendency for more reconstruction phases to be present at concentrations of 1: 12000 and higher but it was impossible to test this statistically because of the small numbers involved.

Cells in control cultures were mostly of typical fibroblast form with long cytoplasmic processes and oval nuclei containing usually two nucleoli. Near the transplant cells were smaller in size and much greater in numbers than toward the periphery. There were always present a certain number of small darkly staining nuclei which probably represent pyknoses due to overcrowding. Treatment at concentrations up to 1: 48000 seems to have little effect upon the appearance of the cells but higher concentrations cause considerable vacuolization. At concentrations of 1: 12000 and stronger, fatty vacuolization becomes very evident and many multinucleate cells are to be seen. This suppression of cyto-

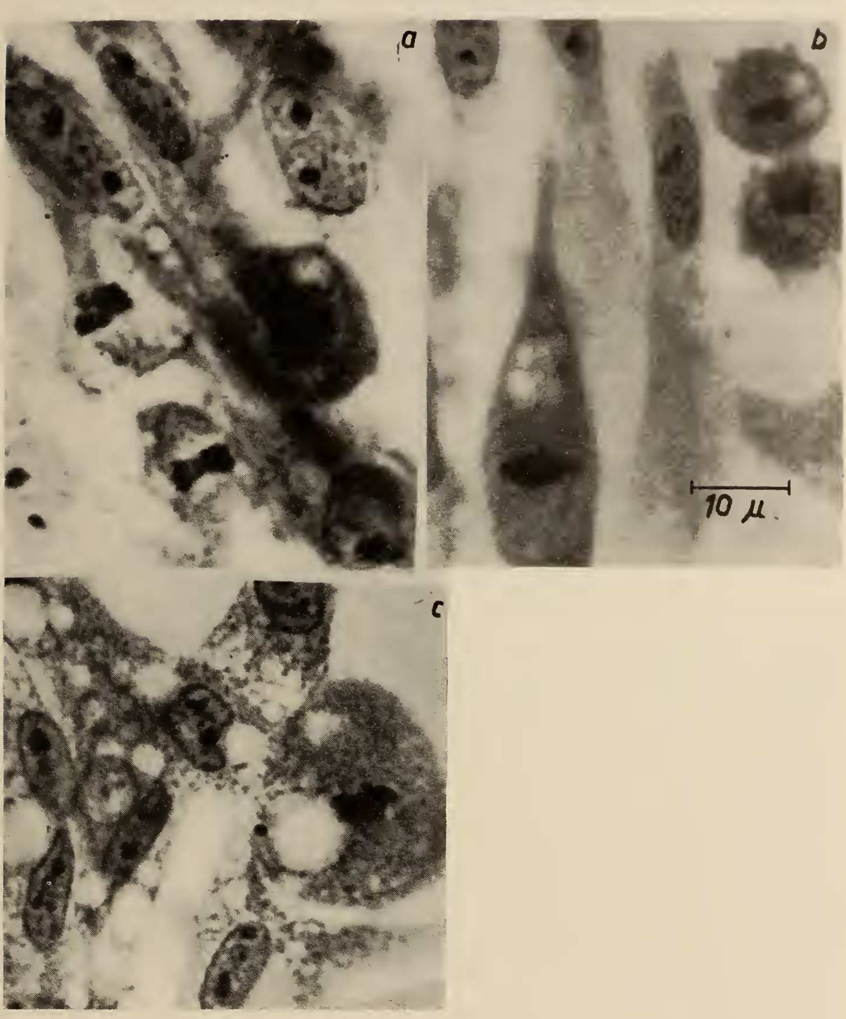


FIG. 18.

Cells from tissue cultures after 48 hours of growth. *a* = control, *b* = treated with ketone 9 at 1: 1500, *c* = treated with ketone 9 at 1: 750. In *b* 2 normal mitoses may be seen despite cytoplasmic vacuoles. In *c* the cytoplasmic vacuolization is very heavy and the cells form a stringy syncitium. Note clear mitotic figure despite presence of vacuoles and small but normal appering nuclei.

plasmic division and occurrence of nuclear fragmentation seems to be a secondary characteristic of the amino ketones and of a different nature than that found by Huber (1947) in *Tubifex* eggs after treatment with benzo- and naphtochinone. At concentrations of 1: 6000, 1: 3000, and 1: 1500 the cells assume a " stringy " syncytial form or network with smaller nuclei, many of which are pyknotic, nucleoli also small and less in number, and with much fatty vacuolization. Although all cells show the effects of treatment, there are still to be found a few normal appearing mitosis in the peripheral zones. Even heavily vacuolized cells still show normal mitotic figures (see figure 18). Addition of amino ketone solutions of 1: 750 to the cultures often completely inhibits growth (see also Lehmann und Dettelbach 1952).

Table 3. — *Mean 48 hour area indexes of amino ketone treated heart tissue cultures.*

	Cont.	1: 48T	1: 24T	1: 12T	1: 6T	1: 3T	1: 1500	1: 750
Ketone 5 (9/2/51)	5.8	5.8	8.0	9.5	6.8	5.8	6.3	0.9
Ketone 5 (26/7/51)	6.15	4.93	4.02	5.24	3.45	4.62	4.97	1.34
Ketone 5 (24/8/51)	5.89	5.31	4.80	6.53	6.34	5.23	5.15	2.66
Ketone 5 (Fresh) (6/9/51)	12.75	—	6.09	—	9.24	—	5.7	1.86
Ketone 5 (Aged) (6/9/51)	12.75	—	7.24	—	8.83	—	6.55	3.10
Ketone 9 (22/8/51)	5.97	6.11	5.64	5.23	5.85	6.54	5.51	1.27
Ketone 9 (14/10/50)[1]	2.5	2.3	1.8	1.68	1.56	1.39	1.17	1.14
Ketone 9 (19/10/50) Aorta tissue	12.9	11.7	10.5	7.8	7.1	5.41	3.98	—
Ketone 9 (Fresh) (29/8/51)	8.84	—	7.6	—	7.27	—	5.33	3.43
Ketone 9 (Aged) (29/8/51)	8.82	—	5.23	—	4.54	—	3.73	2.92

[1] These area indexes were calculated as heavy growth/implant and not as total growth/implant.

C. Effects Upon Cell Migration (Surface areas reduced).

An analysis of cell division only, in tissue cultures, gives an incomplete picture of the effects of a substance upon growth. (See Parker, *op. cit.*, Chap. XIII.) Cellular migration is an important and independent factor in the growth of tissues *in vitro* and is expressed as increase in surface area of the culture. As Cunningham and Kirk (1942) have shown, an exact and complete expression of growth of tissue cultures may only be obtained by measuring volume, density, mitoses, cell migration, etc. Sanford (1951) has also reported a method for measuring proliferation in tissue cultures by enumeration of cell nuclei. In evaluating the effects of the amino ketones upon cells *in vitro*, exact measurement of growth *per se* was considered secondary as long as one aspect of growth (cell migration) was found to be consistently and significantly affected. Cell migration is of course related to cell division, but it is independent of it (Gaillard, 1942) and may be affected by substances which do not effect mitosis. Gaillard and Veer (1948), for example, found that adrenochrome affected cell migration but had little effect upon mitosis in fibroblast cultures. As the amino ketones were also found to have very little direct effect upon mitosis, a study of their effects upon cell migration is of special interest.

Table 3 shows a tabulation of the results of surface measurements of all cultures treated with amino ketone 5 and 9. Each value represents the mean of 3 to 8 separate cultures. It may be seen that growth is not always of the same intensity in the control cultures. On the whole the results show that cultures with a high growth coefficient have a high sensitivity to the amino ketones while the series with lower coefficients are only affected by the strongest concentrations used. This appears to be an analogous relationship to regeneration intensity and sensitivity as found for the regenerating *Xenopus* tail.

Both of the substances either inhibit cell migration strongly or prevent growth altogether at 1: 750. Lehmann and Dettelbach (1952) have published photographs of tissue cultures from one experiment which have been treated with ketone 9 and show the decrease of 48 hour surface area at stronger concentrations.

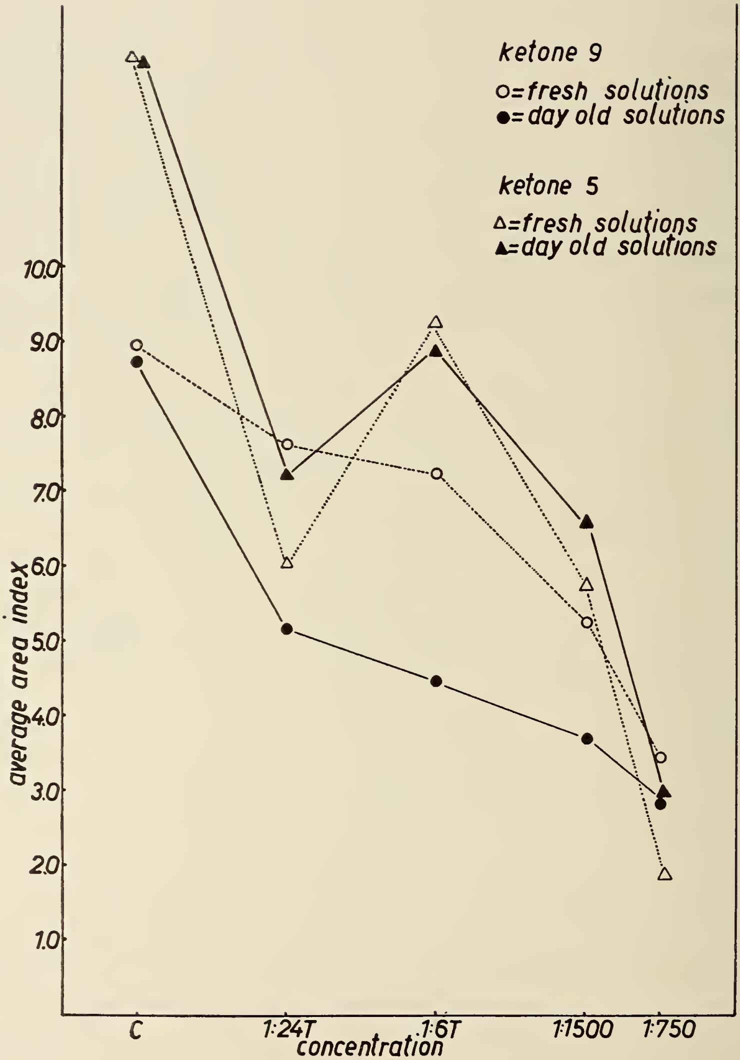


Fig. 19.

Comparison of the effects of fresh and aged solutions of ketones 9 and 5 on cell migration. After ageing ketone 9 is more effective while no difference is found for ketone 5.

As it seemed probable that some oxidation product of the amino ketones is the actual inhibitor (see Section I) it was decided to compare the effects of aged solutions with fresh solutions upon the cultures. For these experiments half of the solutions were made up 24 hours before the begin of the experiments in sterile Tyrode solution and allowed to stand on the light until used. The other half of the amino ketone solutions were made up immediately preceeding their application to the cultures. The tissue upon which the experiments were performed was identical for both halves of the experiment. The results of surface measurements upon these cultures are graphed in figure 19. Amino ketone 9 shows a greater effectiveness in inhibiting cell migration when aged than when fresh but ketone 5 had about the same effectiveness whether fresh or aged. These experiments thus show one more quantitative difference between the two substances.

The action of the amino ketones on tissue cultures can only be incompletely characterized on the basis of our preliminary experiments. The results do show however that weaker concentrations are only effective upon strongly growing fibroblast cultures; primarily upon cell migration and to a lesser degree upon cell division. These findings are strikingly analogous to the action upon the regenerating tissues of *Xenopus* where the migration phase and not the mitotic phase is affected. (See section III *c*, 3.) Further, at no time are mitotic abnormalities found although cytoplasmic vacuolization may by very heavy and nuclear size reduced. Tissue cultures which have been treated at concentrations of 1 : 3000 and stronger show a reduction in number (from two to one) and size of the *nucleoli*. A similar condition is found in the notochord and epidermis of the regenerating *Xenopus* tail after treatment. It is possible that protease synthesis and activity may be disturbed in tissue cultures as it might in the regenerating tail. It is interesting to note in this connection that tumor cells *in vitro* show a high proteolytic and glycolytic capacity (Santesson, 1935; Bucher, 1940) and that cathepsin and peptidase from cancer tissues seem to be identical with enzymes from embryonic cells (Maschmann and Helmert, 1933). In any case further detailed studies of the action of the amino ketones upon the tissues of higher vertebrates seems warranted.

V. SUMMARY

A. Methods.

1. Two amino ketones which are structurally similar to α- leucine, with either a methyl or ethyl group substituted for the end OH group, significantly inhibit regeneration of the *Xenopus* larva tail. (Erlenmeyer and Lehmann, 1949.) These amino ketones are easily oxidized and it is probable that their oxidation products are the actual inhibitors. A detailed study was undertaken on larvae of *Xenopus laevis* to determine as far as possible the mode of action of the amino ketones upon regeneration.

2. The tadpoles were narcotized (MS 222) and 4 mm. amputated from the tail tip. They were then transferred to the test solutions where they were kept for 10 days at 18° C. Five animals were used per 150 cc. container and a geometrically graded series of concentrations from 1: 1000 to 1: 1000000 made up with distilled water was employed.

3. As pre-experimental conditions of temperature, feeding, etc. may influence regeneration intensity considerably all larvae were treated alike and only larvae from the same egg batch were used for one experiment.

4. The length of the regenerating tail was measured on the 5th and 10th days after amputation.

B. Morphological types of reaction.

5. General effects of the amino ketones are contraction of epidermal pigment cells, sluggishness of the tadpoles, and reduction of total size by 8 to 10% after 1: 32000 treatment. Treatment at concentrations of 1: 64000 or weaker limits the effects practically wholly to the regenerating tail.

6. The response to treatment may be classified into three main types corresponding to those found by Lüscher after colchicine treatment. Type I is delayed normal regeneration and varies in length from 50% of the control regeneration up to control

lengths. Type II is chiefly epidermal regeneration with suppression of notochord and spinal cord and varies from 0.3 to 1.0 mm. Type III is complete inhibition of regeneration and is only found after treatment at 1: 8000 or stronger.

7. Type I is most frequent of the types after treatment at 1: 8000 or weaker. The frequency of type II and III increases at concentrations stronger than 1: 8000. Types II and III do not regenerate after treatment for as long as two months unless a new amputation wound is made.

8. The three types are examples of realization grades; i.e. there is not a harmonious reduction of the size of the whole anlage but a total suppression of some parts of the tail. The effect is brought about by a graduated sensitivity of the different regeneration blastemas; the notochord being the most sensitive and probably the most important in tail regeneration.

C. Quantitative Effects Upon Regeneration.

9. The *concentration-inhibition curve is S shaped* and may be simplified by three straight lines (Bretscher). The upper level is determined by control regeneration length. From a concentration of 1: 250000 to 1: 8000 there is a progressive increase in inhibition proportional to increase in concentration (descending arm of curve). Regression lines were calculated for this part of the curve. On the lower levels (less than 1.0 mm.) inhibition is maximal, constant, and independent of concentration and set by animals of the lowest realization grades.

10. *Group susceptibility*, which is the mean inhibition produced by treatment in one series, increases as regeneration intensity (as shown by control regeneration) increases.

11. *Threshold concentration* (determined graphically) varied from group to group between 1: 1000000 and 1: 32000. It decreases as regeneration intensity increases.

12. Groups with higher regeneration intensity are therefore more sensitive to treatment (probably because of their higher metabolic functional condition). Lower regeneration intensity is accompanied by higher threshold concentrations.

13. The tadpoles are most sensitive to amino ketone treatment during the first three days after amputation (phase I) and longer treatment causes little increase in inhibition. The action of the substances is therefore *phase-specific.*

14. *Pre-treatment* at 1: 32000 for 10 days causes a lowering of the regeneration intensity and regeneration is accordingly less at each post-treatment concentration. The slope of the concentration-inhibition curve is not changed, i.e., there is no cumulative effect.

15. Raising the *temperature* to 27° C during the experiments causes no change in the effects of the inhibiting substances although regeneration is accelerated.

16. The *toxicity* of the amino ketones is relatively low and is reached at 1: 2000 to 1: 1000. At 1: 8000 or weaker there are never any lethal effects found.

17. Due to the low toxicity and rather gradual slope of the concentration-inhibition curve, the amino ketones have a large *effective range.* The effective range is greater when regeneration intensity is greater (because of lowered threshold).

D. Histostatic and cytostatic effects.

18. Two series each of ketone 5 and ketone 9 tadpole tails (more than 90 animals) which had been treated at 1: 16000 were fixed after 12, 24, 36, 48, 72, 96 and 120 hours plus controls for each period. These were sectioned and studied.

19. The normally active *epidermis* is strongly affected during the first three days of treatment (phase I). Organization is poor, the nuclei misshapen and the nucleoli reduced in number. Ketone 9 is more effective on the epidermis than ketone 5. The effects are visible till about 120 hours after treatment at 1: 16000.

20. The formation of a mesenchyme area between the tip epidermis and the notochord blastema is disturbed. After treatment a compact mass of tissue is found which may act as a physical block to regeneration. Possibly the formation or activation of proteolytic enzymes which are important in early phases of regeneration is inhibited or suppressed by the amino ketones.

21. Amino ketone treatment causes a reduction of size and number of the *notochord blastema* cells. Organization is also poor and the effects are most pronounced during the first three days. Nucleolar number and size is also reduced. The reduced size of the notochord blastema is probably responsible for the reduced length of the regenerating notochord (lower realization grades).

22. The begin of mitotic activity is delayed in the stump tissues after treatment, more so by ketone 9 than by ketone 5. Mitotic figures are quite normal after treatment and all stages are found. The amino ketones therefore only affect cell division indirectly and are not mitotic poisons.

23. The size of the *nuclei* of the *notochord* blastema and tip *epidermis* is significantly reduced after treatment. Ketone 5 causes a greater reduction at 36 hours and ketone 9 a greater reduction at 48 hours. The *nucleoli* of the notochord blastema cells and tip epidermis are reduced in size and number, or absent completely after treatment.

24. The amino ketones appear to have a strong histostatic action upon interphasic cells, reducing the synthetic intensity or capacity of the cells and the number of cells entering into mitoses.

E. EFFECTS UPON EMBRYONIC CHICK FIBROBLASTS.

25. The amino ketones were further tested on embryonic chick fibroblasts grown in tissue cultures. Mitotic indexes were calculated and surface areas measured of the treated and control cultures. A geometrically decreasing series of concentrations of 1: 48000 to 1: 750 was used.

26. The mitotic coefficient was found to vary greatly but was about within the same range for all cultures even after strong treatment had caused vacuolization of the cells. Normal appearing mitoses are found even after very strong treatment at 1: 750.

27. *Cell migration* is significantly affected by the amino ketones. Culture series with high growth coefficients have a high sensitivity to the amino ketones while series with low coefficients are only affected by the strongest concentrations.

28. As it is probable that some oxidation product of the amino ketones is the actual inhibitor, aged solutions were tested on the cultures. Solutions of *ketone 9 aged for one day* proved to be *more effective* in inhibiting cell migration than fresh solutions but *aged solutions of ketone 5 are not more effective* than fresh solutions.

29. The action of the amino ketones upon tissue cultures are strikingly similar to the effects upon the regenerating *Xenopus* tail. Cell division is practically unaffected while cell migration is inhibited in both cases. Nuclear and nucleolar size and number are reduced in both the regenerating tail and in tissue cultures, and *Xenopus* larvae with a high regeneration intensity as well as tissue cultures with a high growth coefficient are both more sensitive to treatment and have a lower threshold concentration.

VI. LITERATURE

1939. Adova, A. N. and Feldt, A. M.: *Biochemical peculiarities of various regions of Axolotls body connected with form-building under the action of organizers.* Comptes Rend. (Dok.) URSS, *25*, 43-45.

1951. Abei, H.: *Unpublished.*

1948. Andres, G.: *Realisationsgrade bei der Entwicklung des Amphibienlabyrinths.* Arch. Jul. Klaus-Stiftung, *23*, 562-568.

1949. Andres, G., Bretscher, A., Lehmann, F. E., und Roth, D.: *Einige Verbesserungen in der Haltung und Aufzucht von Xenopus laevis.* Experientia, *5*, 83-84.

1932. Arey, L. B.: *Certain basic principles of healing.* Anat. Rec. *51.*

1932. Avel, M.: *L'influence du niveau de section de la corde dorsale sur la longueur du régénérat dans la queue des têtards d'Amphibiens anoures.* Comptes Rend. Soc. Biol., 109, 417.

1939. —— *L'inhibition de la régénération de la corde dorsale chez les têtards des Amphibiens anoures.* Compt. Rend. Soc. Biol. *109*, 693.

1947. Bernhard, W.: *Regenerationshemmung und Auslösung epithelialer Wucherungen durch Colchicin am Schwanz von Ranalarven.* Rev. suisse Zool. *54*, 714-757.

1947. Bretscher, A.: *Reduktion der Zehenzahl bei Xenopuslarven nach lokaler Colchicinbehandlung.* Rev. suisse Zool. *54*, 273-279.

1949. —— *Die Hinterbeinentwicklung von Xenopus laevis Daud. und ihre Beeinflussung durch Colchicin.* Rev. suisse Zool. *56*, 34-93.

1951. —— und Tschumi, P.: *Gestufte Reduktion von chemisch behandelten Xenopus-Beinen.* Rev. suisse Zool. *58*, 391-398.

1951. Bourne, G. H. (ed.): *Cytology and Cell Physiology.* Oxford Univ. Press.

1946. Brachet, J. *Aspects Biochimiques de la Régénération.* Experientia, *2*, 41-48.

1947. —— *Embryologie Chimique.* Masson & Cie., Paris.

1934. Bromley, N. W. und Orechowitch, W. N.: *Ueber die Proteolyse in den regenerierenden Geweben.* Biochem. Zeits., *272*, 324-331.

1933. Brunst, V. V. und Scheremetieva, E. A.: *Untersuchung des Einflusses von Röntgenstrahlen auf die Regeneration beim Triton.* I. *Beobachtungen der Regeneration der Extremitäten beim Triton nach Bestrahlung mit verschiedenen Dosen von Röntgenstrahlen.* Roux Archiv., *128*, 181.

1940. Bucher, O.: *Gewebezüchtung.* Ciba Zeits., *74*, 2530-2568.

1947. —— *Cytopharmakologische Untersuchungen an Gewebekulturen in vitro.* Vierteljahresschrift naturforsch. Ges. Zürich, *92*, 221-238.

1933. Butler, E. G.: *The effects of X-radiation on the regeneration of the fore limb of Amblystoma larvae.* Journ. Exp. Zool. *65*, 271-303.

1935. —— *Studies on limb regeneration in X-rayed Amblystoma larvae.* Anat. Rec., *62*, 295-307.

1941. Caspersson, T.: *Studien über den Eiweissumsatz der Zelle.* Naturwiss. *29*, 33-43.

1947. —— *The relations between nucleic acid and protein synthesis.* Symp. Soc. Exp. Biol. *1*, 127-151.

1940. —— and Schultz, J.: *Ribonucleic acids in both nucleus and cytoplasm and the function of the nucleolus.* Proc. Nat. Acad. Sci. *26*, 507-515.

1944. Clément-Noël, H.: *Les acides pentosenucleiques et la régénération.* Ann. Soc. Roy. Zool. Belg. *75*, 25-33.

1942. Cunningham, B. and Kirk, P. L.: *Measure of " growth " in tissue culture.* Journ. Cell. Comp. Physiol. *20*, 343.

1947. Dalcq, A.: *Recent experimental contributions to brain morphogenesis in amphibians. Sixth Growth Symp.*, 85-119.

1944. Deanesly, R. and Parkes, A. S.: *The preparation and biological effects of iodinated proteins.* B. *Use of Xenopus tadpoles for assay of thyroidal activity.* Journ. Endocrinol. *4*.

1948. Erlenmeyer, H.: *Les composés isostères et le problème de la ressemblance en chimie.* Bull. Soc. Chim. Biol. *30*, 792-805.

1949. —— und Kühne, H.: *Zur Kenntnis des 1-(p-oxyphenyl)-2-amino-butanons-(3).* Helv. Chim. acta. *32*, 370-371.

1949. —— und Lehmann, F. E.: *Regenerationshemmende Wirkung einer leucinähnlichen Verbindung des 2-Methyl-4-amino-hexanon-(5).* Exper. *5*, 472.

1950. von Euler, H.: *Reduktone.* Enke Verlag, Stuttgart.

1951. Friedenwald, J. S.: *The action of nitrogen mustards and related substances on cell division.* Ann. New York Acad. Sci. *51* (Art. 8), 1432-1442.

1942. Gaillard, P. J. *Regularities in the growth of tissue cultures. Chap. III of Causal and Chemical Embryology.* Hermann & Cie, Paris.

1948. —— and Veer, W. L. C. *The influence of adrenochrome on fibroblastic cells in vitro.* Proc. Kon. Nederl. Akad. Wetensch. *51* (no. 7), 1-12.

1944. Gasche, P.: *Metamorphose.* Helv. Physiol. acta *2*, 607-626.

1950. Ghiretti, F.: *On the activity of acid- and alkaline phosphatase during tail regeneration in Triturus cristatus (Lavr.).* Exper. *6*, 98-100.

1944. Gidge, N. M. and Rose, S. M.: *The role of larval skin in promoting limb regeneration in adult Anura.* Journ. Exp. Zool. *97*, 71-93.

1946. Gillette, R. and Bodenstein, D.: *Specific developmentad inhibitions produced in amphibian embryos by a nitrogen mustard compound.* Journ. Exp. Zool. *103*, 1-32.

1928. Godlewski, E.: *Untersuchungen über Auslösung und Hemmung der Regeneration beim Axolotl.* Roux Archiv., *114*, 108-143.

1898. Harrison, R. G. *The growth and regeneration of the tail of the Frog-larva. Studied with the aid of* Borns *method of grafting.* Roux Archiv. *7*.

1932. Helff, O. M.: *Studies on amphibian metamorphosis. X. Hydrogen-ion concentration of the blood of anuran larvae during involution.* Biol. Bull. *63*, 405-418.

1937. —— *The relation of the dorsal nerve cord and notocord to tail regeneration in Anuran larvae.* Anat. Rec. *7* (suppl.), 101-102.

1929. Hellmich, W.: *Untersuchungen über Herkunft und Determination des Regenerations-Materials bei Amphibien.* Roux Archiv. *121*, 135.

1932. Herrick, E. H.: *Mechanism of movement of epidermis, especially its melanophores, in wound healing, and behavior of skin grafts in frog tadpoles.* Biol. Bull. *63*, 271-286.

1945. Hintzsche, E.: *Die Kerngrösse der Follikelepithelien und der Granulosa-Luteinzellen im menschlichen Eierstock.* Mon.-Schr. Geburtshilfe und Gynäkol. *120*.

1949. —— *Zyklische Aenderungen der Kerngrösse in Oberflächenepithel und Drüsen des menschlichen Uterus.* Gynäcol. *128* (Fasc. 4), 270-285.

1947. Huber, W.: *Ueber die antimitotische Wirkung von Naphthochinon und Phenanthrenchinon auf die Furchung von Tubifex.* Rev. suisse Zool. *54*, 61-152.

1936. Ide-Rozas, A.: *Die cytologischen Verhältnisse bei der Regeneration von Kaulquappenextremitäten.* Roux Archiv. *135*, 552-608.

1941. Jacobj, W.: *Die verschiedenen Arten des gesetzmässigen Zellwachstums und ihre Beziehung zu Zellfunktion, Umwelt, Krankheit, maligner Geschwulstbildung und innerem Bauplan.* Roux Archiv. *141*, 584-692.

1933. Jeffimoff, M. I.: *Die Rolle der Haut im Prozess der Regeneration eines Organs beim Axolotl.* Zhurnal. Biol. *2* (russ.), (reviewed by Polezajew and Faworina, 1935).

1949. Kaufmann, S. and Neurath, H.: *Structural requirements of specific inhibitors for α-chymotrypsin.* Journ. Biol. Chem. *181*, 623-633.

1947. Lehmann, F. E.: *Chemische Beeinflussung der Zellteilung.* Exper. *3*, 223.

1946. —— *Einführung in die Physiologische Embryologie*, Verlag Birkhäuser, Basel.

1947. —— *Die Wirkung antimitotischer Stoffe auf das Ei von Tubifex und den regenerierenden Schwanz der Xenopuslarve. Proc. 6th Internat. Cong. Exp. Cytol.*, pp. 156-159.

1948. —— *Realisationsstufen in der Organogenese als entwicklungsphysiologisches und genetisches Problem.* Archiv Jul. Klaus-Stiftung, *23*, 568-573.

1951. —— *Der Kernapparat tierischer Zellen und seine Erforschung mit Hilfe von Antimitotica.* Schw. Zeits. f. Allg. Path. & Bakt. *14*, 487-508.

1945. —— Bernhard, W., Hadorn, H., und Lüscher, M.: *Zur entwicklungsphysiologischen Wirkungsanalyse von antimitotischen Stoffen.* Exper. *1*, 232-233.

1950. —— Bretscher, A., Kühne, H., Sorkin, E., Erne, M., und Erlenmeyer, H.: *Ueber die chemischen und biologischen Eigenschaften einiger α-Aminoketone.* Helv. Chim. Acta. *33*, 1217.

1951. Lehmann, F. E. und Weber, R.: unpublished.

1952. —— und Bretscher, A.: *Wirkungsanalyse regenerationshemmender Stoffe mit Hilfe statistischer Methoden.* Helv. Physiol. et Pharm. Acta. *10*, 20-41.

1952. —— und Dettelbach, H. R.: *Histostatische Wirkungen von Aminoketonen auf die Schwanzregeneration der Xenopuslarve.* Rev. suisse Zool. *59*, 253-295.

1950. Lettré, H.: *Ueber Mitosegifte.* Ergebnisse Physiol. *46*, 379-457.

1945. Linder, A.: *Statistische Methoden.* Birkhäuser Verlag, Basel.

1946 *a*. Lüscher, M.: *Die Hemmung der Regeneration durch Colchicin beim Schwanz der Xenopus-larve und ihre entwicklungsphysiologische Wirkungsanalyse.* Helv. Physiol. Acta. *4*, 465-494.

1946 *b*. —— *Die Wirkung des Colchicins auf die an der Regeneration beteiligten Gewebe im Schwanz der Xenopuslarve.* Rev. suisse Zool. *53*, 683-734.

1933. Maschmann, E. und Helmert, E.: *Ueber Kathepsin und Peptidasen in carcinomatösen und sarkomatösen Tieren.* Zeits. Physiol. Chem., *216*, 161-172.

1924. Naville, A.: *Recherches sur l'histogenèse et la régénération chez les Batraciens Anoures (corde dorsale et téguments).* Arch. de Biol. *34*, 235.

1950. Needham, J.: *Biochemistry and Morphogenesis.* Cambridge Univ. Press.

1948. Ochsé, W.: *Die Zucht des südafrikanischen Krallenfrosches Xenopus laevis Daudin.* Gynaecol. *126*, 57-77.

1938. Okada, Yô, K.: *Regeneration of the tail in Urodeles, with especial remarks on the role of the spinal cord.* Annot. Zool. Japan, *17*, 339-350.

1928. Okuneff, N.: *Ueber einige physiko-chemische Erscheinungen während der Regeneration. I. Mitteilung. Messung der Wasserstoffionenkonzentration in regenerierenden Extremitäten des Axolotls.* Biochem. Zeits. *195*, 421-427.

1929. Okuneff, N.: ..... *II. Mitteilung: Ueber die Pufferung der Gewebe einer regenerierenden Axolotlextremität.* Biochem. Zeits. *212*, 1-15.

1933. —— *Ueber einige physiko-chemische Erscheinungen während der Regeneration.* Biochem. Zeits. *257*, 242-244.

1934. Orechowitsch, W. N. und Bromley, N. W.: *Die histolysierenden Eigenschaften des Regenerationsblastems.* Biol. Zbl. *54*, 524-535.

1938. Parker, R. C. *Methods of Tissue Culture.* Hoeber Inc. New York.

1935. Poležajew, L. W. (unter Beteiligung von W. N. Faworina): *Ueber die Rolle des Epithels in den anfänglichen Entwicklungsstadien einer Regenerationsanlage der Extremität beim Axolotl,* Roux-Arch. *133*, 701-726.

1936. —— Biol. Zhurn. USSR, *15* (Referred to in Needham, *op. cit.*).

1933. —— Biol. Zhurn. USSR, *2* (Refered to in Needham, *op. cit.*).

1947. Pollister, A. W. and Ris, H.: *Nucleoprotein determination in cytological preparations.* Cold spring harbor sympos. Quant. Biol. *12*, 147-157.

1936. Puckett, W. O.: *The effects of X-radiation on limb development and regeneration in Amblystoma.* Journ. Morph. *59*, 173-214.

1950. Risler, H.: *Kernvolumenänderungen in der Larvenentwicklung von Ptychopoda seriata SCHRK.* Biol. Zentralbl. 69, 11-28.

1948. Robertis, E. D. P., Nowinski, W. W., and Saez, F. A.: *General Cytology.* W. B. Saunders Co. Philadelphia.

1948. Rose, S. M.: *Epidermal de-differentiation during blastema formation in regenerating limbs of Triton viridescens.* Jour. Exp. Zool. *108*, 337.

1946. Rosin, S.: *Ueber Bau und Wachstum der Grenzlamelle der Epidermis bei Amphibienlarven.* Rev. suisse Zool. *53*, 133-201.

1947. Roetheli, A.: unpublished.

1940. Ryvkina, D. E.: *Der Gehalt an reduzierten Stoffen in den Geweben bei der Regeneration.* Comptes Rend. Acad. Sci. L'URSS (Doklady), *27*, 380-387.

1939 *a*. —— and Striganova, A. *On the tissues metabolism at wound healing.* (English summary.) Bull. Acad. Sci. L'URSS (Série Biol.), *3*, 445-454.

1939 *b*. —— and Striganova, A.: *Local and general changes in proteolysis during the process of regeneration of an organ.* (English summary.) Bull. Acad. Sci. L'URSS (Série Biol.), *5*, 789-798.

1951. Sanford, K. K.: *The measurement of proliferation in tissue cultures by enumeration of cell nuclei.* Journ. Nat. Canc. Inst. *11*, 773-795.

1935. Santesson, L.: *Characteristics of epithelial mouse tumor cells in vitro and tumor structures in vivo. A comparative study.* Acta Path. et Microbiol. Scand. Suppl. *24*.

1936. Scheremetieva, E. A. und Brunst, V. V.: *Untersuchung des Einflusses von Röntgenstrahlen auf die Regeneration der Extremitäten beim Axolotl. I. Beobachtungen der Regeneration nach Bestrahlung mit verschiedenen Dosen.* Ber. d. Wiss. Biol. *37*, 181.

1933. —— und ——. *Untersuchung des Einflusses von Röntgenstrahlen auf die Regeneration des Schwanzes bei den Kaulquappen von Pelobates fuscus. I. Untersuchung der Regenerate von mit verschiedenen Dosen einmalig bestrahlten Kaulquappen.* Roux Arch. *130*, 771-791.

1951. Stich, H.: *Experimentelle karyologische und cytochemische Untersuchungen an Acetabularia mediteranea.* Zeits. f. Naturforschung, *6 b*, 319-326.

1948. Snedecor, G. W.: *Statistical Methods.* Iowa State Coll. Press, Ames Iowa.

1948. Stowell, R. E.: *Nucleic acids and cytologic changes in regenerating rat liver.* Arch. Path. *46*, 164-178.

1940 *a*. Striganova, A.: *Die proteolytische Aktivität der Gewebe regenerierender Organe bei verschiedenen Milieubedingungen.* Comptes Rend. (Doklady) Acad. Sci. L'URSS, *27*, 385-387.

1940. —— *Der Einfluss der Aktivatoren auf die proteolytische Aktivität regenerierender Organe.* Comptes Rend. (Doklady) Acad. Sci. L'URSS, *27*, 388-392.

1949. Waechter, H.: *Regeneration eines kleinen Stückes der ventralen Bauchwand bei erwachsenen Molchen.* Roux Arch. *144*, 132-154.

1945. Weisz, P. B.: *The development and morphology of the larva of the south african clawed toad, Xenopus laevis.* Journ. Morph. *77*, *I. The third form tadpole.* 163-192. *II. The hatching and the first-and second-form tadpoles.* 193-217.

1937. Wigglesworth, V. B.: *Wound healing in an insect.* Journ. Exp. Biol. *14*, 364-381.

1951. Yokoyama, H. O. and Stowell, R. E.: *Nucleolar volume changes in the mouse pancreas after repeated pilocarpine injections.* Journ. Nat. Canc. Inst. *11*, 939-945.

1916. Zeleny, C.: *Studies on the factors controlling the rate of regeneration.* Ill. Biol. Monogr. *3*.